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14. ABSTRACT

In recent years, progress has been made in the development of immune-based therapy for cancer. Conceptually, these treatment strategies have the potential of harnessing the immune system to combat and eliminate cancer cells. One major obstacle to the success of immunotherapy in both human and animal studies is the development of immunologic tolerance in tumor-bearing hosts. Therefore, the immune system fails to recognize cancer cells as dangerous and actively suppresses anti-tumor immune responses. Identification of the underlying mechanisms and the critical players that drive tolerance to the tumor is critical to improve the therapeutic efficacy of immunotherapy. Recent data indicate that activin A, a small protein secreted by some immune cells and by breast cancer cells has immune regulatory functions that may play a key role in promoting escape of tumors from immune control. The proposed studies will test the hypothesis that activin A secreted by breast cancer cells plays a key role in suppressing antitumor immunity. The goals are to demonstrate the role of activin A produced by breast cancer cells in tumor growth and metastasis, and the potential therapeutic benefit of blocking activin A to increase the response to radiotherapy.

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1 INTRODUCTION

Owing to its ability to spread systematically, breast cancer remains a life-threatening tumor. Therefore, efforts in developing new treatment strategies are needed in order to eradicate metastatic breast cancer. In this respect, the activation of the immune system to elicit anti-tumor immune responses represents one of the most promising approaches that have recently demonstrated some success in other diseases. However, clinically apparent tumors have already harnessed host mechanisms to prevent immune activation and to induce an immunosuppressive microenvironment hindering immunotherapy-based treatments. As a consequence, the immune system fails to recognize cancer cells as dangerous and actively suppresses anti-tumor immune responses.

Identification of the underlying mechanisms and the critical players that drive tolerance to the tumor is critical to improve the therapeutic efficacy of immunotherapy. Recent data indicate that activin-A, a small protein secreted by some immune cells [1-3] and by breast cancer cells [4], has immune regulatory functions [5-10] that may play a key role in promoting escape of tumors from immune control. The specific hypothesis of this project is that activin-A secreted by breast cancer cells plays a key role in suppressing antitumor immunity. The goals are to demonstrate the role of activin-A produced by breast cancer cells in tumor growth and metastasis, and the potential therapeutic benefit of blocking activin-A to increase the response to radiotherapy (RT).

2 KEYWORDS

Breast cancer, metastasis, transforming growth factor-beta (TGFβ) superfamily, activin-A, radiotherapy (RT), immunosuppression, immunotherapy, induced regulatory T cells (Tregs), anti-tumor immunity, abscopal effect.

3 OVERALL PROJECT SUMMARY

During the first year of this postdoctoral BCRP fellowship, we have demonstrated in vitro that tumor-derived activin-A promotes immunosuppression by inducing a tolerogenic phenotype of the dendritic cells (DCs) as well as partially enhancing the conversion of naïve CD4 T cells into Tregs. More importantly, similarly to TGFβ, our results suggest that breast cancer cells upregulate activin-A production in response to radiation exposure, which could counter the pro-immunogenic effects of radiotherapy.

Since our data support a key role of activin-A in immune tolerance by the tumor, derivatives of 4T1 ($4T1^{shInhba}$) cells transduced with a set of plasmid encoding short-hairpin (shRNA) specific for murine *Inhba* have been prepared during year 2. Several approaches have been tested in order to determine that the pTRIPZ doxycycline inducible lentiviral shRNA vector efficiently knockdown activin-A expression both at the gene and protein levels. Therefore, the role of tumor-derived activin-A in immune response to breast cancer in vivo was investigated in year 3.

AIM1-Task 2c / AIM2-Task 1: *Effect of activin-A blockade in vivo on breast cancer immunity*

To determine the role of activin-A in vivo, we injected s.c. 150,000 $4T1^{shInhba}$ metastatic breast cancer cells or its scrambled negative control ($4T1^{shSCR}$) into BALB/c mice (day 0) and induced the *Inhba* gene knockdown by feeding mice with doxycycline (doxy) at 100ug/mL starting at day 8. Additionally, because we have previously shown that RT induces activin-A

production by tumor cells in vitro, we asked whether blocking activin-A during RT improved therapeutic activity. To that end, RT was delivered to the tumor in 6Gy fractions on five consecutive days beginning day 13. Mice were followed for tumor growth and survival or euthanized three animal per group at day 22 for immune evaluation (Figure 1A).

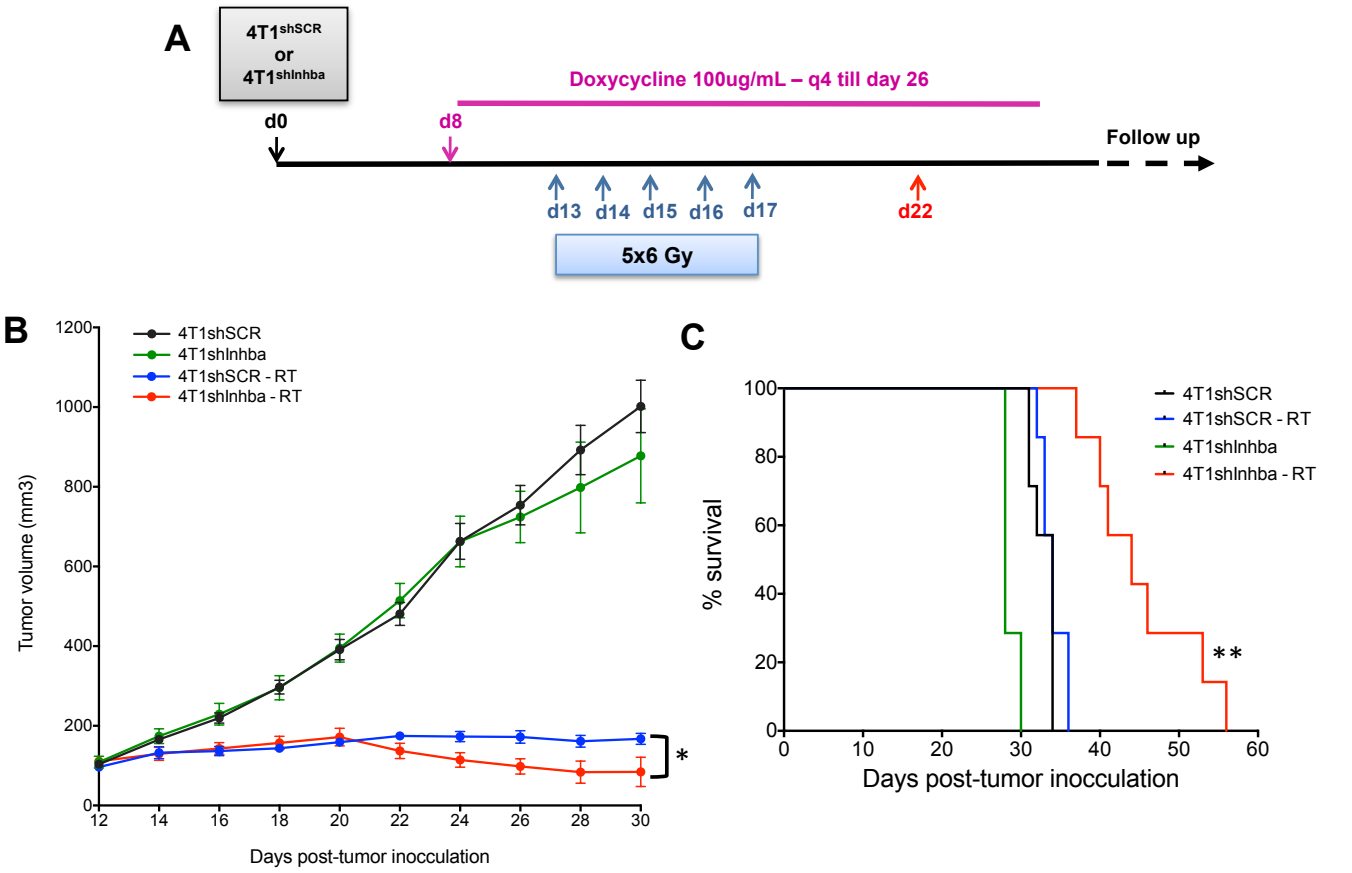

Figure 1: **Blockade of activin A synergizes with local RT to improve tumor control**. (**A**) Experimental plan. Syngeneic immunocompetent BALB/c mice were inoculated with 150,000 4T1^{shSCR} or 4T1^{shInhba} mammary carcinoma cells s.c. on day 0. To induce the knockdown of activin A, mice were fed with doxycycline on day 8 till day 26. RT was delivered to the tumor in 6Gy fractions on five consecutive days beginning day 13. Mice were followed for tumor growth or euthanized at day 22 to collect and digest tumors in order to evaluate phenotypical changes in the immune cells population. (**B**) Tumor growth overtime in 4T1^{shSCR} or 4T1^{shInhba}-tumor bearing mice treated w/o local RT (n=10 per group). Data are representative of two independent experiments. * *p<0.05.* (**C**) Survival of 4T1^{shSCR} or 4T1^{shInhba}-tumor bearing mice treated w/o local RT (n=7/group). Data are representative of two independent experiments. ***p<0.005*.

Results revealed that activin-A knockdown itself did not have any effect on either growth of the subcutaneous tumor and the survival of the animals. As expected, RT caused a significant growth delay of the irradiated tumor ($p<0.0005$ 4T1^{shSCR} vs 4T1^{shSCR} – RT). However, combining local RT with activin-A blockade significantly improved therapeutic efficiency with better tumor control leading to a marked survival benefit (Figure 1B and 1C). These data indicate that **local RT synergizes with activin-A blockade to improve tumor control and mice survival**.

To determine whether the benefit of combining RT and activin-A knockdown is a result of the activation of the immune system, we first restimulated in vitro tumor-draining lymph nodes (TDLN) with gp70 (or AH1A5 peptide), which is derived from an endogenous retrovirus, to study cross-priming of CD8 T cells ex vivo [11]. Data indicated that T cells producing IFNγ in response to gp70 recognition were detected only in the TDLN of mice treated with RT in combination with

activin-A inhibition (Figure 2A); therefore suggesting that **priming of T-cell responses to an endogenous tumor antigen is promoted by local radiotherapy but is blocked by radiation-induced tumor-derived activin-A**.

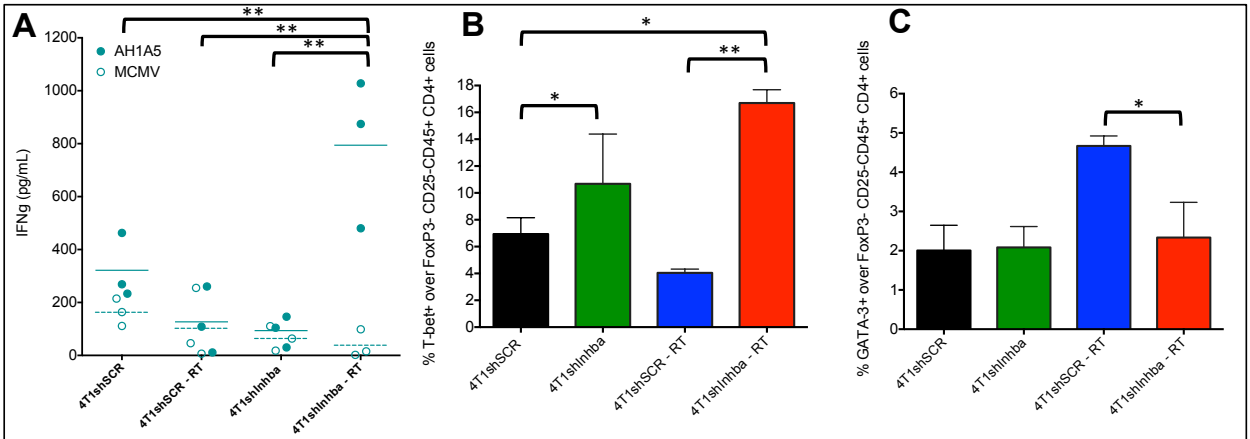

Figure 2: **Activin A gene knockdown with local RT induces CD8+ T-cell responses to endogenous tumor antigen and promotes Th1 polarization of CD4+ T cells in vivo** . (**A**) IFNγ production by CD8+ T cells from lymph node (LN) draining 4T1 tumors in response to peptide derived from gp70 (AH1A5, closed circles) or irrelevant peptide (MCMV, open symbols)/. Each symbol represents one animal. Horizontal lines indicates the mean od the antigen-specific (solid lines) or control (dashed lines) IFNγ concentration. Data are representative of two independent experiments. ***p< 0.005.* (**B-C**) Bars graphs of T-bet (B) and GATA-3 (C) expression over the CD4+ T cells subset. Results are expressed as mean ± SD and are representative of two independent experiments. **p<0.05.*

It has been shown that activin-A can influence immune cells function [5-10] by downregulating the T-box transcription factor (T-bet), a helper type 1 (Th1) specific transcription factor and a hallmark of Th1 cell-mediated immunity [12, 13]. Moreover, reports indicated that activin-A can be produced by CD4+ T cells of the helper type 2 (Th2) phenotype, suggesting that activin-A produced by breast cancer cells contributes to creating an immunosuppressive tumor microenvironment (TME) by promoting the skewing of CD4+ T cells towards a Th2 phenotype [14]. Therefore, to study whether tumor-derived activin-A impair Th1 cell-mediated anti-tumor immunity by fostering a Th2 phenotype of CD4+, we collected and digested the 4T1-irradiated tumors to investigate the polarization of CD4+ tumor-infiltrated lymphocytes (TILs) by staining for GATA binding protein 3 (GATA-3), a master transcription factor involved in Th2 development [15, 16]

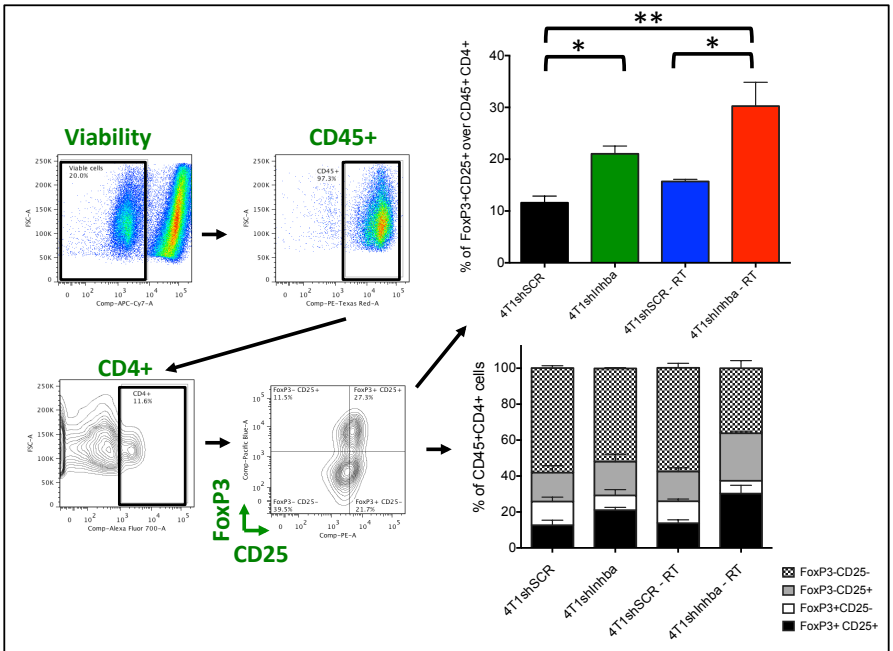

Figure 3: **Blockade of activin A promotes the recruitment of intratumoral regulatory T cells in vivo**. (**A**) Representative dot plots. (**B-C**) Bar graphs show mean percentage of CD45+ CD4+ T cells expressing FoxP3 and CD25 markers. Data are representative of two independent experiments. ** p<0.05. **p<0.005*

and for T-bet (Figures 2B-2C). Data revealed a significant increased of T-bet together with a decrease of GATA-3 expression; especially after local RT exposure; of alive CD45+ CD4+ TILs only when the ability of breast cancer cells to produce activin-A is inhibited.
This suggests that **tumor-derived activin-A impairs breast cancer anti-tumor immune responses by; at least; promoting Th2 polarization in CD4+ TILs subset**.

We have previously demonstrated that tumor-derived activin-A promotes immunosuppression by enhancing the conversion of naïve CD4+ T cells into regulatory T cells (Tregs) in vitro.
However, surprisingly, while activin-A blockade enhanced RT-induced T cells priming (Figure 2), it did **increase rather than reduce intratumoral regulatory T cells in vivo** (Tregs, defined as CD4+ CD45+ CD25+ FoxP3+) (Figure 3). These findings could explain why despite some evidence of anti-tumor immunity, the majority of 4T1 tumors in mice treated RT and activin-A inhibition did not undergo complete regression and cure (Figure 1).

Activin-A and transforming growth factor-beta (TGFβ) display overlapping activities including the ability to promote Tregs [17]. We have recently shown that in situ vaccination by local tumor irradiation is hindered by activation of latent TGFβ [18]. Because there is evidence of a compensatory mechanism between TGFβ and activin-A signaling [19], **we asked whether the increase of Tregs in mice treated with RT and activin-A knockdown (Figure 3) is a result of the concomitant TGFβ activation by RT, therefore dampening the benefit of either activin-A or TGFβ blockade during RT.**

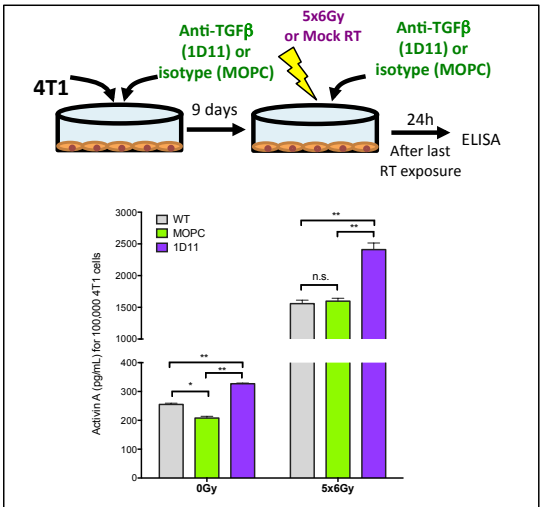

Figure 4: **Prolonged TGFβ inhibition and RT further increase the secretion of activin A**. 4T1 breast cancer cells were cultured with anti-TGFβ monoclonal antibody for 9 days. Cells were then irradiated with 5 fractions of 6Gy. 24hrs after the last RT exposure, supernatant were collected to quantify activin A secretion by ELISA. Results are expressed in mean ± SD. Duplicate experiment. **p<0.05; **p<0.005.*

To first confirm that activin-A and TGFβ exhibited an intertwined biology that could further contribute to creating a pro-tumorigenic immunological environment in the 4T1-tumor, we first study the ability of 4T1 breast cancer cells to produce activin-A in response to prolonged TGFβ inhibition.
Results confirmed our hypothesis hereby **activin-A and TGFβ cross-regulate each other with a significant over-secretion of 4T1-derived activin-A exposed to prolonged inhibition of TGFβ; effect that is emphasized after RT exposure** (Figure 4).

To determine whether concomitant inhibition of TGFβ and activin-A promote radiation-induced immune responses against breast cancer, we injected 150,000 $4T1^{shInhba}$ cells or its scrambled negative control ($4T1^{shSCR}$) into BALB/c mice and induced the *Inhba* gene knockdown by feeding mice with doxycycline (doxy) at 100ug/mL starting at day 8. TGFβ neutralizing 1D11 or isotype control (MOPC) antibodies were given i.p. every other day starting on day 12. RT was delivered to the 4T1 tumor in 6Gy fractions on five consecutive days beginning on day 13 (Figure 5A). Mice were followed for tumor growth or euthanized at day 22 to harvest TDLN for ex vivo restimulation and tumors to evaluate Tregs infiltration.

Neither in vivo 1D11 nor activin-A gene knockdown by themselves affected tumor growth. However, each intervention significantly improved tumor control achieved by RT. TGFβ blockade in mice bearing irradiated $4T1^{ShInhba}$-tumors did not further improve tumor control

(Figure 5B) but did significantly improved IFNγ production by CD8+ T cells in response to a tumor-specific antigen (Figure 6) as well as mice survival (Figure 5C).

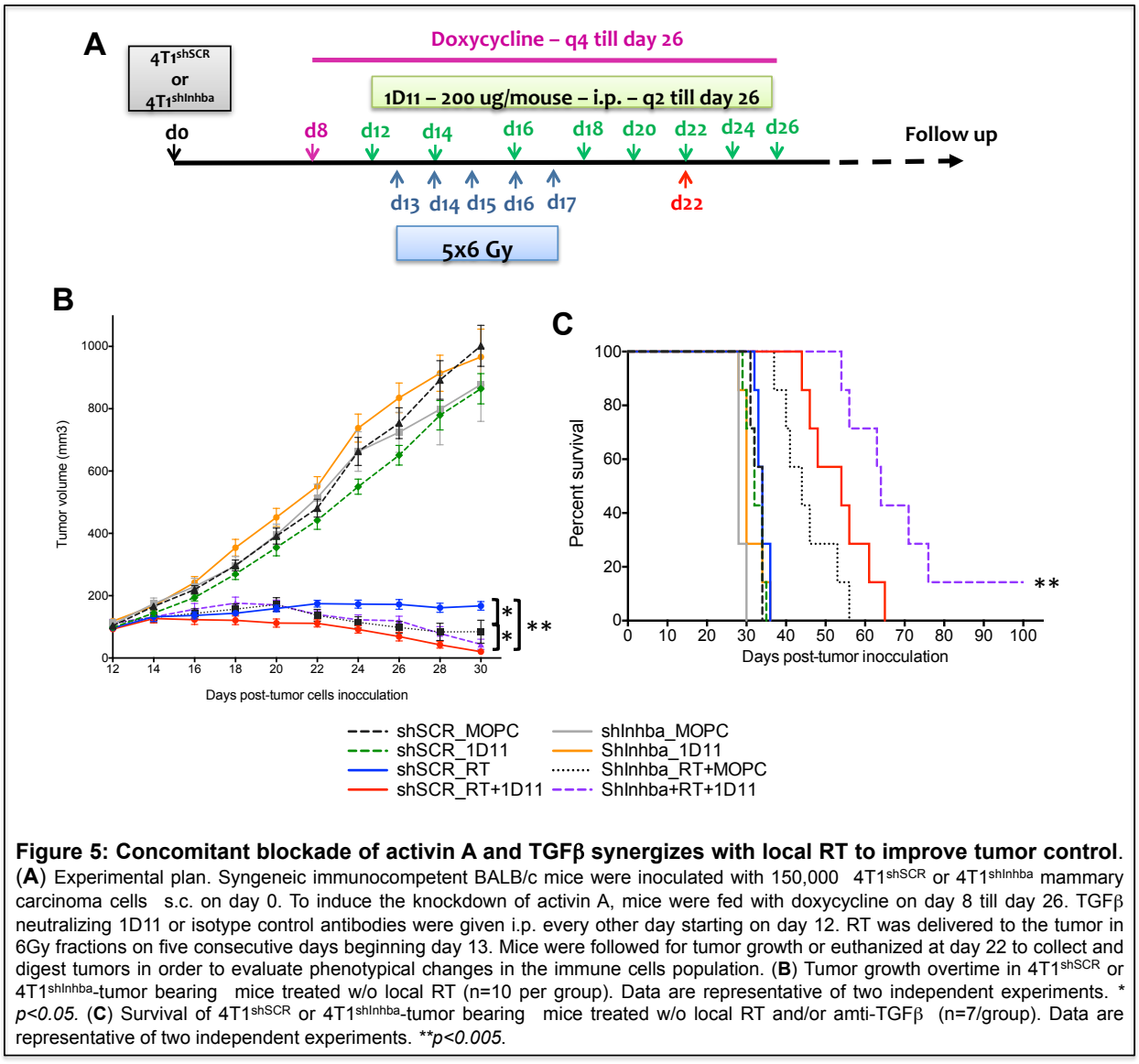

Figure 5: Concomitant blockade of activin A and TGFβ synergizes with local RT to improve tumor control. (**A**) Experimental plan. Syngeneic immunocompetent BALB/c mice were inoculated with 150,000 4T1^{shSCR} or 4T1^{shInhba} mammary carcinoma cells s.c. on day 0. To induce the knockdown of activin A, mice were fed with doxycycline on day 8 till day 26. TGFβ neutralizing 1D11 or isotype control antibodies were given i.p. every other day starting on day 12. RT was delivered to the tumor in 6Gy fractions on five consecutive days beginning day 13. Mice were followed for tumor growth or euthanized at day 22 to collect and digest tumors in order to evaluate phenotypical changes in the immune cells population. (**B**) Tumor growth overtime in 4T1^{shSCR} or 4T1^{shInhba}-tumor bearing mice treated w/o local RT (n=10 per group). Data are representative of two independent experiments. * *p<0.05.* (**C**) Survival of 4T1^{shSCR} or 4T1^{shInhba}-tumor bearing mice treated w/o local RT and/or amti-TGFβ (n=7/group). Data are representative of two independent experiments. ***p<0.005.*

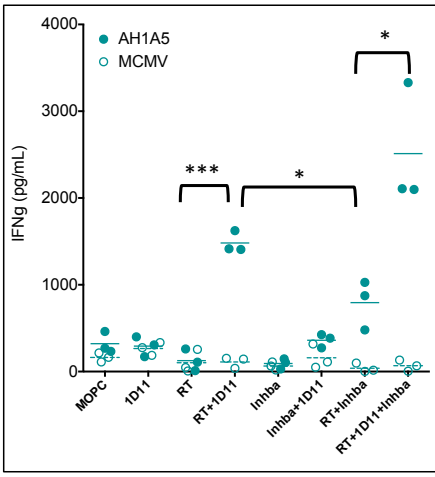

Figure 6: **TGFβ inhibition and Activin A gene knockdown synergize with local RT to enhance CD8+ T-cell responses to endogenou tumor antigen**. IFNγ production by CD8+ T cells from lymph node (LN) draining 4T1 tumors in response to peptide derived from gp70 (AH1A5, closed circles) or irrelevant peptide (MCMV, open symbols)/. Each symbol represents one animal. Horizontal lines indicates the mean od the antigen-specific (solid lines) or control (dashed lines) IFNγ concentration. Data are representative of two independent experiments. *p<0.05, ***p< 0.005,* ****p<0.0005.*

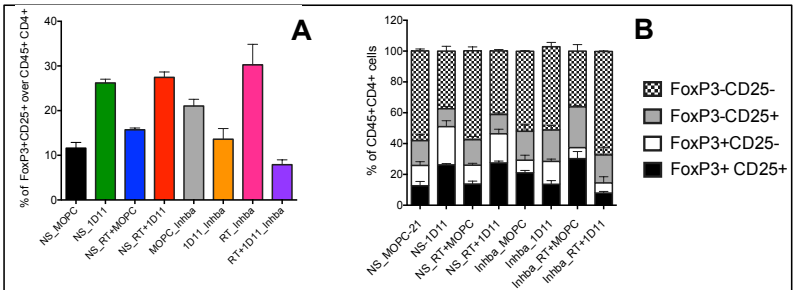

Figure 7: **Blockade of activin A and TGFβ reduce the recruitment of intratumoral regulatory T cells in vivo**. (**A**) Representative dot plots. (**B**) Bar graphs show mean percentage of CD45+ CD4+ T cells expressing FoxP3 and CD25 markers. Data are representative of two independent experiments. ** p<0.05. **p<0.005*

As expected, inhibiting TGFβ or activin-A increased intratumoral Tregs (Figure 7). As described by other, RT alone icreased Tregs [20]; increase that was markedly larger in the presence of 1D11 or activin A knockdown, In marked contrast, when both TGFβ and activin-A were inhibited, Tregs numbers significantly decreased in irradiated tumors below baseline (Control: 11.6%; 1D11: 26.2%, shInhba: 21%; 1D11+shInhba: 13.6%; RT: 15.7%; RT+1D11: 27.5%; RT+shInhba: 30.3%; RT+1D11+shInhba: 7.9% of Tregs).

These data suggest **a complex regulation of Tregs in the tumor by TGFβ and activin-A. Combined TGFβ blockade and activin-A during RT may be required to improve immune-mediated rejection of irradiated breast tumors**.

4 KEY RESEARCH ACCOMPLISHMENTS

- Demonstrated that knockdown activin-A in breast cancer cells result in therapeutic benefit.
- Determined that activin-A is a regulator of the priming of T cells against tumor-derived antigen released by radiotherapy.
- Demonstrated that activin-A increase rather than reduced the recruitment of intratumoral regulatory T cells in vivo.
- Obtained data suggesting that activin-A and TGFβ cross-regulate each other to further contribute to creating a pro-tumorigenic immunological environment in breast cancer.
- Demonstrated that concomitant inhibition of activin-A and TGFβ reduces intratumoral regulatory T cells, improves priming and mice survival.

5 CONCLUSION

Overall, the pre-clinical data obtained in the third year of the fellowship strongly support the hypothesis that activin-A produced by breast cancer cells is involved in breast cancer immune escape by promoting a Th2 tumor microenvironment. They also highlight the critical role of activin-A in suppressing the development of immune responses in context of radiotherapy.

Importantly, results highlight a complex intertwined biology between activin-A and TGFβ signaling; suggesting that combined inhibition of activin-A and TGFβ during radiotherapy may be required to optimize the ability of RT to induce in situ tumor vaccination and anti-tumor immune responses capable to achieve systemic control of metastatic disease (abscopal effect).

Improved understanding of the biology of TGFβ superfamily members in the context of radiotherapy will allow us to identify additional actionable targets to improve response to treatment. We plan to explore in the next year the role of activin-A in the TSA breast cancer tumor model to confirm the above-described findings. We have previously demonstrated that TSA cells are low producers of activin-A, as a consequence, to determine whether tumor-derived activin-A is responsible for breast cancer immune escape, we will use a set of

tetracycline knock-in vectors (pTRIPZ, Dharmacon) to overexpressed activin-A in TSA cells. We expect to enhance immunosuppression by TSA-breast cancer tumors by notably promoting the recruitment of intratumoral regulatory T cells therefore confirming our pre-clinical data obtained in the 4T1 breast cancer tumor model.

In the past year, I have actively participated in the departmental works-in-progress seminars and attended to New York University (NYU) immunology club presentations as well as NYU Cancer Institute Breast Biology Working Group sessions, which have enriched my knowledge in cutting edge research in breast cancer. After moving to Weill Cornell Medicine (WCM) in September 2015, I have attended to Radiation Oncology and Immunology seminars that have improved my understanding of radiation-induced anti-tumor immunity. I also have had the opportunity to attend to the NYC Tumor Immunology Radiation Oncology (TIRO) meetings held every other month at WCM to foster inter-institutional exchange and collaboration in the field of radiation oncology and cancer immunology. I have had the great opportunity to attend to the President's Research Seminar Series (PRSS) organized by the Memorial Sloan Kettering Cancer Institute every Wednesday, to improve my knowledge of tumor immunology and cancer research. I have met with leaders in the field of breast cancer immunology at recent meetings, and will continue to work closely with my mentor, Dr Sandra Demaria, who I meet every week to discuss results and plan experiments. She continues to be an invaluable resource to my training as a future breast cancer scientist.

6 PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS

a. Manuscripts submitted for publication:

1. Lay Press:

Nothing to report.

2. Peer-Reviewed Scientific Journals:

- Vanpouille-Box C, Diamond JM, Pilones KA, Zavadil J, Babb JS, Formenti SC, Barcellos-Hoff MH and Demaria S. *TGFβ is a master regulator of radiation therapy-induced antitumor immunity*. **Cancer Research**. 2015 1;75(11):2232-42.
  (APPENDIX 1)

- Vanpouille-Box C, Pilones KA, Wennerberg E, Formenti SC and Demaria S. *In situ vaccination by radiotherapy to improve responses to anti-CTLA-4 treatment*. **Vaccine**. 2015 16;33(51):7415-22.
  (APPENDIX 2)

3. Invited Articles:

Nothing to report.

4. Abstracts:

- Vanpouille-Box C, Aryankalayil M, Pilones KA, Formenti SC, Coleman N and Demaria S. *Fractionation but not single dose radiation is an optimal adjuvant for in situ tumor vaccination*. **2015 AACR Annual Meeting**, USA, Philadelphia, April 18-22 2015.
  (APPENDIX 3)

- Vanpouille-Box C, Formenti SC and Demaria S. *Cooperative effects of TGFβ and activin A control regulatory T cells numbers in irradiated tumors*. **CRI-CIMT-EATI-AACR – The inaugural International Cancer Immunotherapy Conference: Translating Science into Survival**, USA, New York, September 16-19 2015.
  (APPENDIX 4)

- Vanpouille-Box C, Formenti S and Demaria S. Radiation-induced DNA-damage response drives the secretion of activin A by tumor cells fostering immunosuppression in breast cancer. **2015 Radiation Research Society 61st annual meeting**, USA, Weston, September 19-22 2015.
  (APPENDIX 5)

- Vanpouille-Box C, Formenti S and Demaria S. *TGFβ and activin A control regulatory T cells in irradiated tumors*. **2015 SITC 30th Annual Meeting**, USA, New Harbor, November 4-8 2015.
  (APPENDIX 6)

b. National Meeting and Presentations:

- 2015 American Association of Cancer Research (AACR) Annual Meeting – 18-22 April 2015, Philadelphia, USA. *Poster Presentation.*
- 2015 CRI-CIMT-EATI-AACR – The inaugural International Cancer Immunotherapy Conference: Translating Science into Survival, USA, New York, September 16-19 2015. *Poster Presentation.*
- 2015 Radiation Research Society 61st annual meeting, USA, Weston, September 19-22 2015. *Poster Presentation.*
- 2015 SITC 30th Annual Meeting, USA, New Harbor, November 4-8 2015. *Poster Presentation.*

7 INVENTIONS, PATENTS AND LICENSES

Nothing to report.

8 REPORTABLE OUTCOMES

Nothing to report.

9 OTHER ACHIEVEMENTS

Awards:

- 2015 AACR-Susan G Komen Scholar-in-Training Award.
- 2015 Scholar-in-Training Travel Award from the Radiation Research Society
- 2015 Scholar-in-Training Travel Award from the Society for Immunotherapy of Cancer.

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11 APPENDICES

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Microenvironment and Immunology

Cancer Research

TGFβ Is a Master Regulator of Radiation Therapy-Induced Antitumor Immunity

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Abstract

T cells directed to endogenous tumor antigens are powerful mediators of tumor regression. Recent immunotherapy advances have identified effective interventions to unleash tumor-specific T-cell activity in patients who naturally develop them. Eliciting T-cell responses to a patient's individual tumor remains a major challenge. Radiation therapy can induce immune responses to model antigens expressed by tumors, but it remains unclear whether it can effectively prime T cells specific for endogenous antigens expressed by poorly immunogenic tumors. We hypothesized that TGFβ activity is a major obstacle hindering the ability of radiation to generate an *in situ* tumor vaccine. Here, we show that antibody-mediated TGFβ neutralization during radiation therapy effectively generates $CD8^+$ T-cell responses to multiple endogenous tumor antigens in poorly immunogenic mouse carcinomas. Generated T cells were effective at causing regression of irradiated tumors and nonirradiated lung metastases or synchronous tumors (abscopal effect). Gene signatures associated with IFNγ and immune-mediated rejection were detected in tumors treated with radiation therapy and TGFβ blockade in combination but not as single agents. Upregulation of programmed death (PD) ligand-1 and -2 in neoplastic and myeloid cells and PD-1 on intratumoral T cells limited tumor rejection, resulting in rapid recurrence. Addition of anti–PD-1 antibodies extended survival achieved with radiation and TGFβ blockade. Thus, TGFβ is a fundamental regulator of radiation therapy's ability to generate an *in situ* tumor vaccine. The combination of local radiation therapy with TGFβ neutralization offers a novel individualized strategy for vaccinating patients against their tumors. *Cancer Res; 75(11); 2232–42. ©2015 AACR.*

Introduction

Recent progress in cancer immunotherapy has demonstrated that unleashing the power of tumor-reactive T cells with immune checkpoint inhibitors is an effective treatment in a substantial percentage of patients with metastatic melanoma, renal cell, and lung cancer (1–3). However, the majority of cancer patients do not respond to these treatments due to the presence of multiple immunosuppressive mechanisms or the absence of tumor-reactive T cells. Therapeutic cancer vaccines have shown some success in inducing T cells specific for tumor antigens, but their efficacy is limited by the intrinsic genomic instability of tumors generating highly mutable targets (4). Thus, a strategy to induce effective antitumor immunity should combine an individualized vaccination approach with targeted interventions to overcome immunosuppression.

Experimental data and clinical observations indicate that radiation therapy (RT) has the potential to convert the irradiated tumor into an *in situ* vaccine (5). Data in mouse tumors expressing OVA or other model antigens have shown that RT induces cross-priming of T cells to these relatively strong antigens and that T cells contribute to the therapeutic effect of RT (6, 7). Rejection of irradiated tumors is facilitated by RT-induced modulation of chemokines and cell surface molecules that enhance T-cell recruitment (8, 9) and the interaction of CTLs with tumor cells (10–12). RT-elicited cross-priming of tumor-specific T cells depends on generation of the molecular signals that define an immunogenic cell death (13) and requires type I IFN production by infiltrating immune cells (14). However, rejection of nonirradiated metastases and synchronous tumors is usually not achieved by RT alone (15–17) and, despite the widespread use of RT in cancer treatment, the clinical response of metastases outside of the radiation field (abscopal effect) is an extremely rare occurrence (18).

These observations suggest that generation of a tumor vaccine by RT may be impeded by other radiation effects. Of particular concern is the activation of TGFβ (19). The latter is mediated by RT-induced reactive oxygen species that cause a conformational change of the latency-associated peptide–TGFβ complex releasing active TGFβ (20, 21). We have previously shown that activated TGFβ reduces radiosensitivity of tumor cells by promoting the DNA damage response (22). Importantly, TGFβ is a powerful immunosuppressive cytokine that hinders cross-priming of T cells by impairing the antigen-presenting function of dendritic cells and the functional differentiation of T cells into effectors (23).

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We hypothesized that TGFβ may be a major obstacle to the optimal activation of antitumor T-cell responses by RT. Here, we show that TGFβ neutralizing antibodies administered during RT uncover the ability of RT to induce T-cell responses to endogenous tumor antigens in preclinical models of metastatic breast cancer. Importantly, only the combination of RT with anti-TGFβ, but not each treatment alone, induced T-cell–mediated rejection of the irradiated tumor and nonirradiated metastases in mice, indicating that blocking TGFβ unleashes the potential of RT to generate an *in situ* tumor vaccine. Although adaptive immune resistance that developed in responding tumors limited the efficacy of this approach, it could be overcome by additional blockade of the immune checkpoint receptor PD-1.

Materials and Methods

Mice

Six-week-old BALB/c female mice from Taconic Animal Laboratory were maintained under pathogen-free conditions in the animal facility at NYU Langone Medical Center (New York, NY). All experiments were approved by the Institutional Animal Care and Use Committee.

Cells and reagents

4T1 and TSA cells were obtained from F. Miller, Michigan Cancer Foundation, Detroit, MI (24) and P.L. Lollini, University of Bologna, Bologna, Italy (25), respectively. Cells were authenticated by morphology, phenotype, growth, and pattern of metastasis *in vivo* and routinely screened for *Mycoplasma*. Cells were cultured in DMEM (Invitrogen Corporation) supplemented with 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 2.5×10^{-5} mol/L 2-mercapthoethanol, and 10% FBS (Life technologies). 1D11, a pan-isoform, TGFβ-neutralizing mouse mAb that binds only to active TGFβ (26) or 13C4 isotype mAb was provided by Genzyme Inc. Anti–PD-1 RMP1-14 mAb was purchased from BioXCell.

Tumor challenge and treatment

4T1 model: mice were injected subcutaneously (s.c.) in the right flank with 5×10^4 4T1 cells on day 0. TSA model: mice were injected s.c with 1×10^5 TSA cells in the right flank (primary tumor) on day 0, and in the left flank (secondary tumor) on day 2. Perpendicular tumor diameters were measured with a Vernier caliper, and tumor volumes calculated as length $\times$ width$^2 \times 0.52$. On day 12, when tumors reached 60 to 80 mm^3, mice were randomly assigned to treatment groups. RT was delivered to the tumor volume as previously described with some modifications (16). Briefly, all mice (including mice receiving sham radiation) were anesthetized by intraperitoneal (i.p.) injection of avertin (240 mg/kg) and the primary tumors irradiated with 6 Gy on days 13, 14, 15, 16, and 17 using the Small Animal Radiation Research Platform (SARRP Xstrahl Ltd). 13C4 and 1D11 mAbs were administered i.p. (200 μg/mouse) every other day from day 12 to day 28. In some experiments, anti–PD-1 mAb RMP1-14 was injected i.p. (200 μg/mouse) on days 18, 22, 26, and 30.

Depletion of CD4$^+$ and CD8$^+$ T cells was achieved by injecting GK1.5 or 2.43 mAb (BioXCell) given i.p. at 100 μg/mouse on 3 consecutive days, starting at day 10, and was maintained by weekly injections. Depletion was confirmed by staining spleen and tumor-draining lymph node (TDLN) cells with non–cross-reactive FITC-RMA4-4 and PE-anti–CD8β mAb (BD PharMingen).

Flow-cytometric analysis

Single-cell suspensions of collagenase-digested tumors were stained with the following antibodies purchased from eBioscience: fixable viability dye efluor 450, CD69-FITC, PD-1-PE, CD4-PeCy7, CD45-Alexa Fluor 700, CD8a-PE efluor 610, CD40-FITC, CD70-PE, MHC-II IAd-APC, CD11c-PE efluor 610, CD45-APC, CD3-PerCP-Cy5.5, CD11b-PerCP-Cy5.5, EpCAM-PeCy7, PD-L1-PE, and PD-L2-FITC. Phospho-Smad2/3 levels were assessed as previously described (27). Briefly, TDLN cells were stained with anti-mouse CD4-PE and anti-mouse CD8-FITC (eBioscience), fixed, permeabilized (Foxp3 Fixation/Permeabilization Concentrate and Diluent kit, eBioscience), and stained with goat anti-phospho-Smad2/3 (Ser 423/425) followed by APC-labeled donkey anti-goat IgG (Santa Cruz Biotechnology). All samples were acquired with LSRII flow cytometer and analyzed with FlowJo software (version 7.3.6).

Analysis of IFNγ production by CD8 T cells

A total of 5×10^5 TDLN cells were stimulated *ex vivo* with 1 μmol/L of the following peptides (GenScript): AH1A5 (SPSYVYHQF), survivin-1 (GWEPDDNPI), survivin-2 (AFLTVKKQM), Twist (LYQVLQSDEL), pMCMV (YPHFMPTNL; refs. 17, 25, 28, 29). After 72-hour culture in 1 mL T-cell medium (RPMI-1640 medium supplemented with 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 50 μmol/L 2-mercapthoethanol, 10% FBS) supplemented with 10U/mL of human rIL-2, cell-free supernatants were assessed for IFNγ concentration using the FlowCytomix kit (eBioscience).

Genome-wide microarray analysis

Total RNA was purified from 4T1 tumors with Qiagen RNeasy Mini Kit. The 260/280 nm ratio was calculated using Nanodrop ND-1000. RNA integrity of each sample was confirmed by capillary electrophoresis resolving the 18S and 28S ribosomal RNA profile on the Agilent Technologies 2100 Bioanalyzer. Genome-wide microarray analyses were performed with 100 ng of total RNA from three independent biologic replicates per group using Affymetrix mouse genome 430 2.0 arrays (3′ IVT labeling). The data obtained have been deposited in the Gene Expression Omnibus (GEO) database (GSE61208). Affymetrix CEL files were normalized using Robust Multichip Average algorithm (30) in GeneSpring GX software (Agilent Technologies) and each probe was normalized to the median value of the control specimens (Sham+Isotype). Differentially regulated genes were identified by feature selection algorithm Pavlidis template matching (31) using a P value <0.05, to identify gene sets for subsequent pathway analysis. The most altered canonical pathways and gene networks were identified using the Ingenuity Pathway Analysis (IPA) software.

Immunostaining of tumor sections

Tumors were fixed for 1 hour at 4°C in a 4% paraformaldehyde, incubated overnight in 30% sucrose, and frozen in optimum cutting temperature medium. Sections were incubated with 0.1% Tween 20 and 0.01% Triton X-100 for 20 minutes, followed by blocking with 4% rat serum in 4% BSA/PBS and staining with PE-Texas Red–rat anti-mouse CD4 or PE-rat anti-mouse CD8α (Caltag), and 5 μg/mL 4′,6-diamidino-2-phenylindole (Sigma). Images were obtained using a Leica SNC400F fluorescence slide scanner. CD4 and CD8 T cells were counted in five randomly selected (×200) fields in each tumor.

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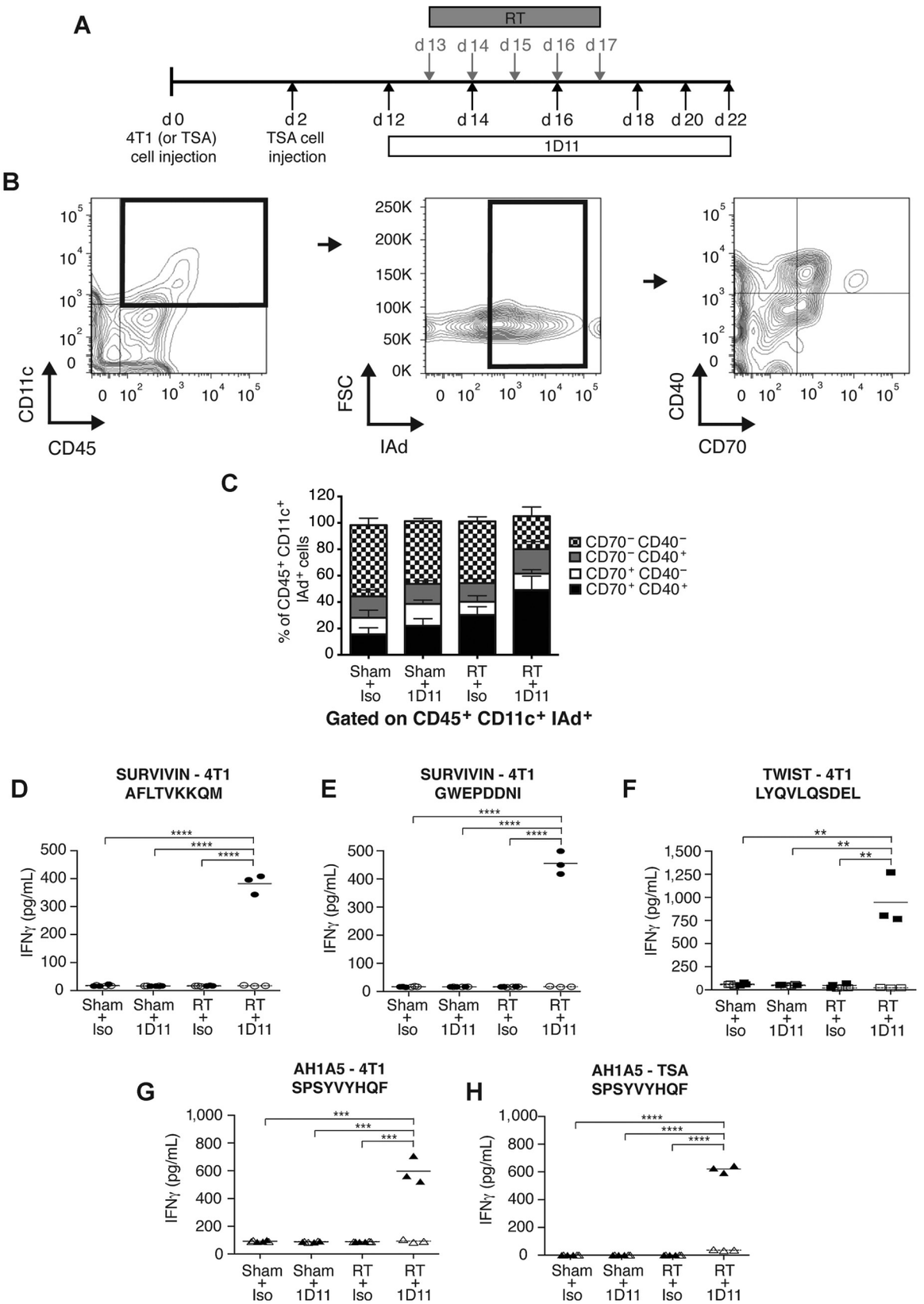

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Statistical analysis

The unpaired Student *t* test was used for analysis of IFNγ levels, cell number, and phenotype. Treatment effect on tumor growth was assessed using random coefficient regression. The dependent variable was the natural log of tumor volume at all available time points. Exact Mann–Whitney tests were used to compare treatment groups in terms of lung metastasis count on day 28. ANOVA based on ranks was used to test the interactions of RT and 1D11 exposure in terms of their impact on lung metastasis count. Survival differences were assessed using log-rank tests. The Kaplan–Meier method was used to estimate median survival times. All reported *P* values are two sided and are declared as significant at the level of 5%. The statistical computations were carried out using SAS for windows, version 9.3 (SAS Institute).

Results

Priming of $CD8^+$ T cells reactive to multiple endogenous tumor antigens by RT requires TGFβ blockade

Radiation has been shown to promote DC activation while TGFβ hinders it (23, 32). To determine whether blocking TGFβ improves RT-induced tumor-infiltrating dendritic cells (TIDC) activation, mice bearing established flank tumors from the 4T1 mouse breast carcinoma received i.p. injection of 1D11, a pan-isoform neutralizing TGFβ mAb or its isotype control starting at day 12 post-tumor cells injection. In half of the mice in each group, tumors were treated with RT given in 5 daily doses of 6 Gy starting on day 13 (Fig. 1A). TIDC, defined as $CD45^+CD11c^+MHC\text{-}II^+$ cells, was analyzed at day 22 for expression of activation markers CD40 and CD70, which plays a critical role in controlling priming versus tolerance induction of $CD8^+$ T cells (33). The percentage of $CD40^+CD70^+$ TIDC was increased by RT and TGFβ blockade used alone (22.3% ± 4.9 in 1D11 and 30.2% ± 6.2 in RT vs. 15.5% ± 4.9 in control, $P < 0.05$ and $P < 0.005$, respectively) but the increase was far larger when they were combined (49.1% ± 10.5 in RT+1D11; $P < 0.0005$ compared with control; $P < 0.005$ compared with 1D11 or RT). Overall, about 80% of TIDC expressed at least one activation marker in mice treated with RT + TGFβ blockade (Fig. 1B and C).

Next, TDLNs were analyzed to assess the effect of RT and TGFβ blockade on priming of 4T1 tumor antigen-specific $CD8^+$ T cells. TGFβ signaling leads to downstream phosphorylation of Smad2/3, which was similarly detected in T cells of untreated mice and mice receiving RT (Supplementary Fig. S1), indicating that baseline production of active TGFβ is sufficient to affect T-cell responses. Administration of 1D11 effectively abrogated TGFβ signaling in T cells of both untreated and RT-treated mice. However, $CD8^+$ T-cell responses to four MHC class I-restricted peptides derived from three endogenous tumor antigens, survivin, Twist, and gp70 (25, 28, 29), were detected only in mice treated with the combination of RT and 1D11 (Fig. 1D–G). The requirement for TGFβ blockade to achieve priming of $CD8^+$ T cells to gp70 was confirmed in the TSA mouse breast carcinoma (Fig. 1H). These data indicate that priming of T-cell responses to endogenous tumor antigens is promoted by tumor irradiation but is blocked by TGFβ.

Inhibition of tumor growth and metastases by the combination of RT and TGFβ blockade

To determine whether priming of tumor-specific T cells by RT and TGFβ blockade was associated with therapeutic activity, we used two experimental settings. First, mice bearing day 12 subcutaneous 4T1 tumors, a time at which this aggressive carcinoma has already metastasized to the lungs (24), were treated as above, but 1D11 administration was continued until day 28 (Fig. 2A). Mice were followed for tumor growth and lung metastases were evaluated at day 28. TGFβ blockade by itself did not have any effect on either growth of the subcutaneous tumor or number of lung metastases (Fig. 2B and C). RT caused a significant growth delay of the irradiated tumor ($P < 0.0005$ RT vs. control) without any effects on lung metastases. However, when RT was given with TGFβ blockade, both the irradiated tumor and nonirradiated lung metastases were markedly inhibited ($P < 0.0005$ RT+1D11 vs. all other groups), demonstrating a synergistic interaction of the two treatments.

In the second experimental setting, 1×10^5 TSA cells were injected subcutaneously on day 0 to generate a "primary" tumor and on day 2 in the contralateral flank to generate a "secondary" tumor. Treatment was started when both tumors were palpable, but only the primary larger tumor was irradiated (Fig. 2A). Similarly to what was observed with 4T1 tumor, TGFβ blockade did not have any effect on either of the two tumors (Fig. 2D and E). When combined with RT, however, TGFβ blockade significantly improved response of the irradiated tumor with 81% showing complete regression compared with only 5% with RT alone ($P < 0.001$). Importantly, growth of the contralateral, nonirradiated tumors was significantly inhibited only when RT was given with TGFβ blockade ($P < 0.001$). Taken together, these data indicate that TGFβ blockade during radiotherapy allows priming of tumor-specific $CD8^+$ T cells to occur, and both improves the local response to RT and elicits abscopal responses.

Gene signatures associated with immune-mediated tissue rejection are upregulated in irradiated tumors only when TGFβ is blocked

TGFβ is a pleiotropic cytokine that promotes tumor growth and metastases by acting on multiple cell compartments, including the neoplastic cells themselves, the tumor stroma, and the immune system (34). Once tumors were established, blocking TGFβ did not impair growth of 4T1 subcutaneous tumors or lung metastases (Fig. 2), suggesting that at more advanced stages, these processes are less dependent on TGFβ. However, TGFβ inhibition also impairs the DNA damage response thereby increasing radiosensitivity of breast and other cancer cells (22, 35). To gain a

Figure 1.

TGFβ blockade with RT enhances TIDC activation and induces $CD8^+$ T-cell responses to endogenous tumor antigens. A, treatment schema. B and C, analysis of 4T1 TIDC at day 22 ($n = 9$/group). To obtain sufficient material, three tumors were pooled to obtain three independent samples for each group. Viable cells were gated on $CD45^+CD11c^+IAd^+$ TIDC and analyzed for expression of activation markers CD40 and CD70. B, representative dot plots. C, bar graphs showing significant increase in mean percentage of TIDC expressing CD40 and CD70 in tumors of mice treated with RT+1D11 ($P < 0.005$ compared with all other groups). D–H, IFNγ production by $CD8^+$ T cells from LN draining 4T1 (D–G) or TSA (H) tumors in response to peptides derived from survivin (D and E, closed circles), Twist (F, closed squares,), and gp70 (AH1A5; G and H, closed triangles), or irrelevant peptide (open symbols). Each symbol represents one animal. Horizontal lines indicate the mean of antigen-specific (solid lines) or control (dashed lines) IFNγ concentration. Data are representative of three independent experiments. **, $P < 0.005$; ***, $P < 0.0005$; ****, $P < 0.00005$.

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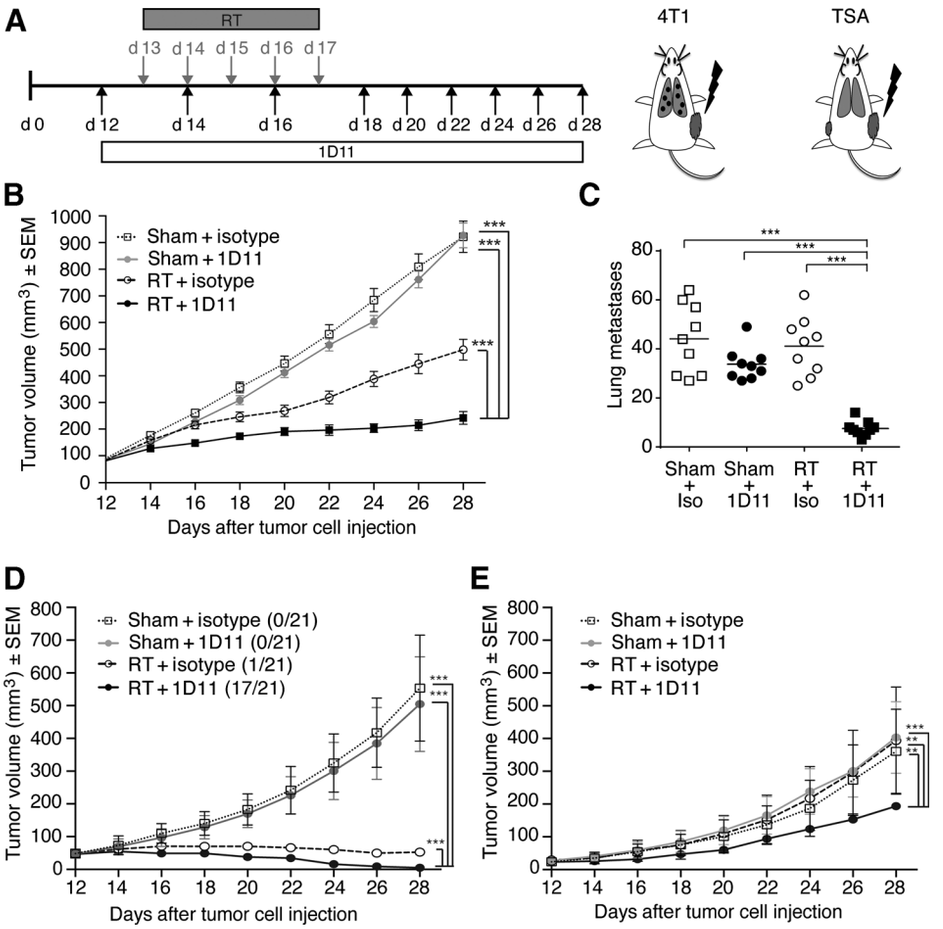

Figure 2.
TGFβ blockade with RT inhibits irradiated tumor and nonirradiated metastases. A, tumor models and treatment schema. B and C, tumor volume overtime (B) and lung metastases quantified at day 28 (C) in 4T1 tumor-bearing mice treated with Sham+Isotype ($n = 9$, open squares), Sham+1D11 ($n = 8$, gray circles), RT+Isotype ($n = 9$, open circles), and RT+1D11 ($n = 9$, black squares). Data are representative of three independent experiments. D and E, tumor volume overtime of primary (right flank; D) and secondary (left flank; E) TSA tumors in mice treated with Sham+Isotype ($n = 21$, open squares), Sham+1D11 ($n = 21$, gray circles), RT (primary only) +Isotype ($n = 20$, open circles), and RT (primary only) +1D11 ($n = 21$, black squares). Data are representative of two experiments. **, $P < 0.005$; ***, $P < 0.0005$.

comprehensive portrait of the effects of RT and TGFβ blockade on gene expression in tumors, we performed microarray analysis of 4T1 tumors 4 days after completion of RT. Using Pavlidis template matching (31), we identified 500 genes selectively upregulated in tumors treated with RT+1D11 (Fig. 3A and B). The enriched dataset was analyzed using IPA software to obtain the molecular pathways differentially expressed in the combination treatment group. The top 20 significantly upregulated canonical pathways were all immune related (Supplementary Fig. S2). Importantly, many of these pathways were found to be upregulated in regressing melanoma metastases from patients responding to immunotherapy (36) and to be associated with a favorable prognosis in human breast tumors (37). Furthermore, the top three self-organizing gene interaction networks generated by IPA indicated activation of a process encompassing the coordinated production of chemokines and cytokines that promote the recruitment of CTLs, and the activation of immune effector function genes and of IFNγ (Fig. 3C). Overall, these data indicate that the dominant change in tumors treated with RT and concomitant TGFβ blockade is activation of immune-mediated tissue rejection pathways (38).

The therapeutic effect of RT in combination with TGFβ blockade is T-cell dependent

To confirm that TGFβ blockade elicited immune-mediated tumor rejection, tumor sections were stained for T-cell markers. Rare T cells were present in untreated 4T1 tumors and their density was not altered significantly by either RT or TGFβ blockade alone. In contrast, when the two treatments were combined, $CD4^+$ and $CD8^+$ T cells infiltrating the irradiated 4T1 tumors markedly increased (Fig. 4A and B). Importantly, a marked increase in tumor-infiltrating T cells was also seen with TGFβ blockade in nonirradiated tumors of mice bearing two TSA tumors, only one of which was irradiated (Fig. 4C), suggesting that immune-mediated tumor rejection occurs systemically. Consistent with this, depletion of $CD4^+$ or $CD8^+$ T cells abrogated regression of the irradiated 4T1 tumors and restored the number of lung metastases in mice treated with TGFβ inhibition + RT (Fig. 4E–G). Overall, these data demonstrate that tumor-specific T cells activated in mice treated with RT and TGFβ blockade are key mediators of regression of irradiated tumors and of the abscopal responses.

Adaptive immune resistance mediated by PD-1 pathway limits the therapeutic efficacy of RT with TGFβ blockade

Despite the prominent T-cell infiltrate, the majority of 4T1 tumors in mice treated with RT and TGFβ blockade did not undergo complete regression, suggesting that additional immunosuppressive mechanisms hinder effector T-cell function in the tumor. Because interactions between the immune checkpoint receptor PD-1 and its ligands PD-L1 and PD-L2 are an important mechanism inhibiting tumor rejection by T cells

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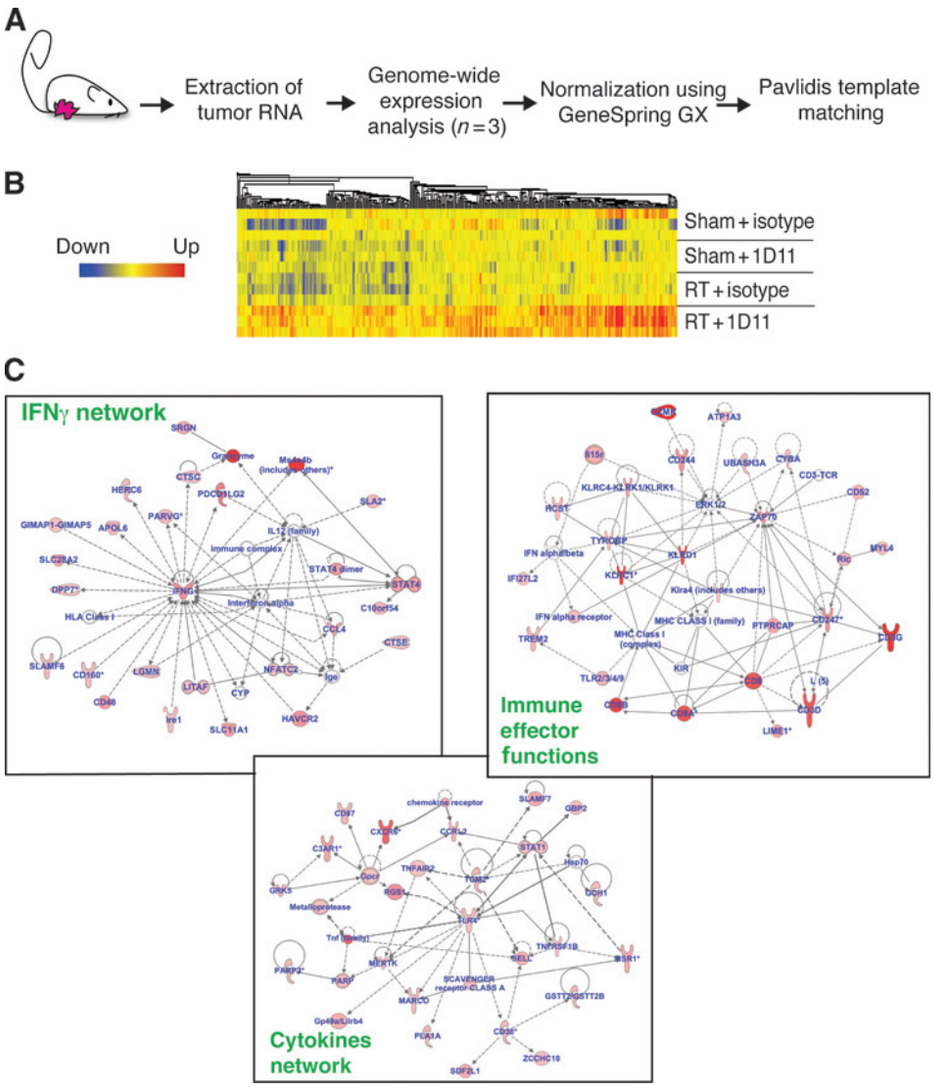

Figure 3.
TGFβ blockade with RT activates immune-mediated tissue rejection pathways. A, experimental schema. Genome-wide analysis of RNA from 4T1 tumor of mice treated as indicated in Fig. 1 and harvested at day 22 ($n = 3$/group). B, heatmap showing the top 500 genes selectively upregulated in RT+1D11 tumors. C, top three self-organizing networks according to IPA representing schematic relationship among genes upregulated in RT+1D11 tumors. Upregulated genes and gene complexes are in red, while no color fill designates genes that are part of the network but not of the gene list. Bold lines indicate direct interaction. Dotted lines indicate indirect interaction.

(39), we next investigated the expression of PD-1 and its ligands in the tumor.

Intratumoral $CD4^+$ and $CD8^+$ T cells showed increased expression of the activation marker CD69 in tumors treated with RT, which was further enhanced by TGFβ blockade (Fig. 5A and B). Interestingly, about half of $CD69^+$ T cells coexpressed PD-1, whereas only a minor subset was PD-1^+CD69^-, suggesting that most PD-1^+ T cells are activated tumor-specific T cells (40). Expression of both PD-L1 and PD-L2 was induced by treatment with RT and TGFβ blockade in a small subset of 4T1 cells, identified as $EpCAM^+CD45^-$ cells, and in a larger subset of intratumoral $CD45^+CD11b^+$ myeloid cells (Fig. 5C and D). Irradiation in the absence of TGFβ blockade had only a minor effect on the expression of PD-L1 and PD-L2, while TGFβ blockade had no effect.

We then tested the hypothesis that treatment with PD-1 blocking antibody could further improve the therapeutic effect achieved with RT and TGFβ blockade (Fig. 6A). Blockade of PD-1 alone or in combination with TGFβ blockade did not have any effect on tumor growth or survival (Fig. 6B and C). RT alone, as expected, caused growth delay of the irradiated tumor without an effect on survival. RT given with either TGFβ or PD-1 blockade improved control of the irradiated tumor with 44% and 25% complete regressions, respectively, and a significant extension of survival ($P < 0.001$ compared with control and RT), but in both groups, all tumors recurred before day 40. In contrast, 75% of the mice treated with RT and blockade of both PD-1 and TGFβ achieved complete regression of the irradiated tumor, and tumor recurrence was delayed, resulting in a significant increase in survival compared with all other groups ($P < 0.001$). ANOVA based on ranks demonstrated a significant interaction among the three interventions ($P = 0.006$), suggesting complementary effects. Interestingly, PD-1 blockade in the presence of RT promoted priming of tumor-specific $CD8^+$ T cells in TDLN, and further enhanced the response achieved with TGFβ blockade (Fig. 6D). Thus, when used with RT, anti–PD-1 may have effects at both effector and priming phase that contribute to tumor rejection. Thus, these data support the

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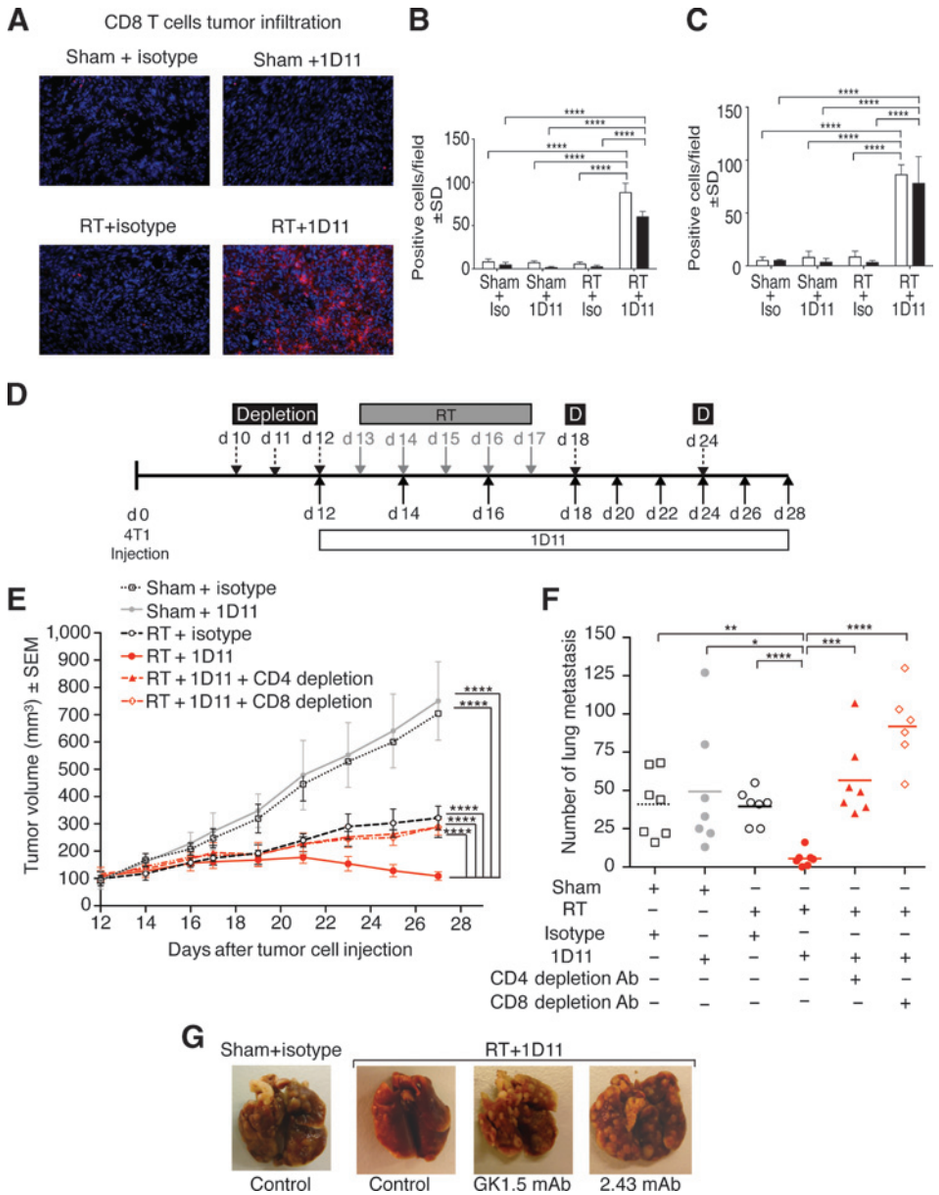

Figure 4.
The therapeutic effect of local RT and TGFβ blockade is T-cell dependent. 4T1 primary (A and B) and TSA secondary (C) tumors of mice treated as indicated in Fig. 1 were harvested at day 22 ($n = 3$/group) and stained for T-cell markers. A, representative fields (×200) showing $CD8^+$ T cells (red). Nuclei were stained with DAPI (blue). Mean number ± SD of $CD4^+$ (white bars) and $CD8^+$ (black bars) cells/field in three mice/group in 4T1 (B) and TSA (C) tumors. D, treatment schema for depletion of $CD4^+$ or $CD8^+$ T cells in 4T1 tumor-bearing mice treated with RT and TGFβ blockade. E and F, tumor volume (E) and lung metastases (F) at day 28 in mice treated with Sham+Isotype (open squares, $n = 7$), Sham+1D11 (gray circles, $n = 7$), RT+Isotype (open circles, dashed black line), RT+1D11 (red circle, red line, $n = 7$), RT+1D11+CD4 depletion (red triangles, dashed red line, $n = 7$), and RT+1D11+CD8 depletion (open red diamond, dashed and dotted red line, $n = 7$). Data are representative of two independent experiments. G, representative photographs of lungs. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$; ****, $P < 0.00005$.

rational targeting of multiple inhibitory pathways in combination with RT.

Discussion

Recent successes of immunotherapy have shown that unleashing the power of the immune system to fight cancer can change the outcome for metastatic disease. Although responses are seen in a relatively small percentage of patients, the durability of the response is far superior to other types of treatment (41, 42). Therefore, combination treatments that can extend the benefits of immunotherapy to more patients are under active investigation. We previously proposed that RT has effects that may complement those of at least some immunotherapies (43), a concept that has recently gained broader support (44, 45). Here, we show that irradiation of tumors unresponsive to targeting of two key immunosuppressive pathways alone, or even in combination, enables effective antitumor immune responses.

Accumulating evidence suggests that the presence of substantial preexisting tumor-specific T cells predicts which patients will respond to immunotherapy (46), emphasizing the inability of most currently tested treatments to effectively induce *de novo* T-cell priming to endogenous tumor antigens. Although RT has been previously shown to prime T cells to model antigens expressed by tumors (6, 7), evidence that it can prime T-cell responses to less immunogenic endogenous tumor antigens has been limited (47). We have uncovered that RT-induced T-cell priming is impeded by radiation-induced TGFβ. TGFβ blockade has effects on multiple immune cells that contribute to tumor control (34). Radiation elicits TGFβ activation (20, 21); notably, our data show that blocking TGFβ in established tumors is insufficient to slow growth of the primary tumor or its lung metastases. Although we cannot

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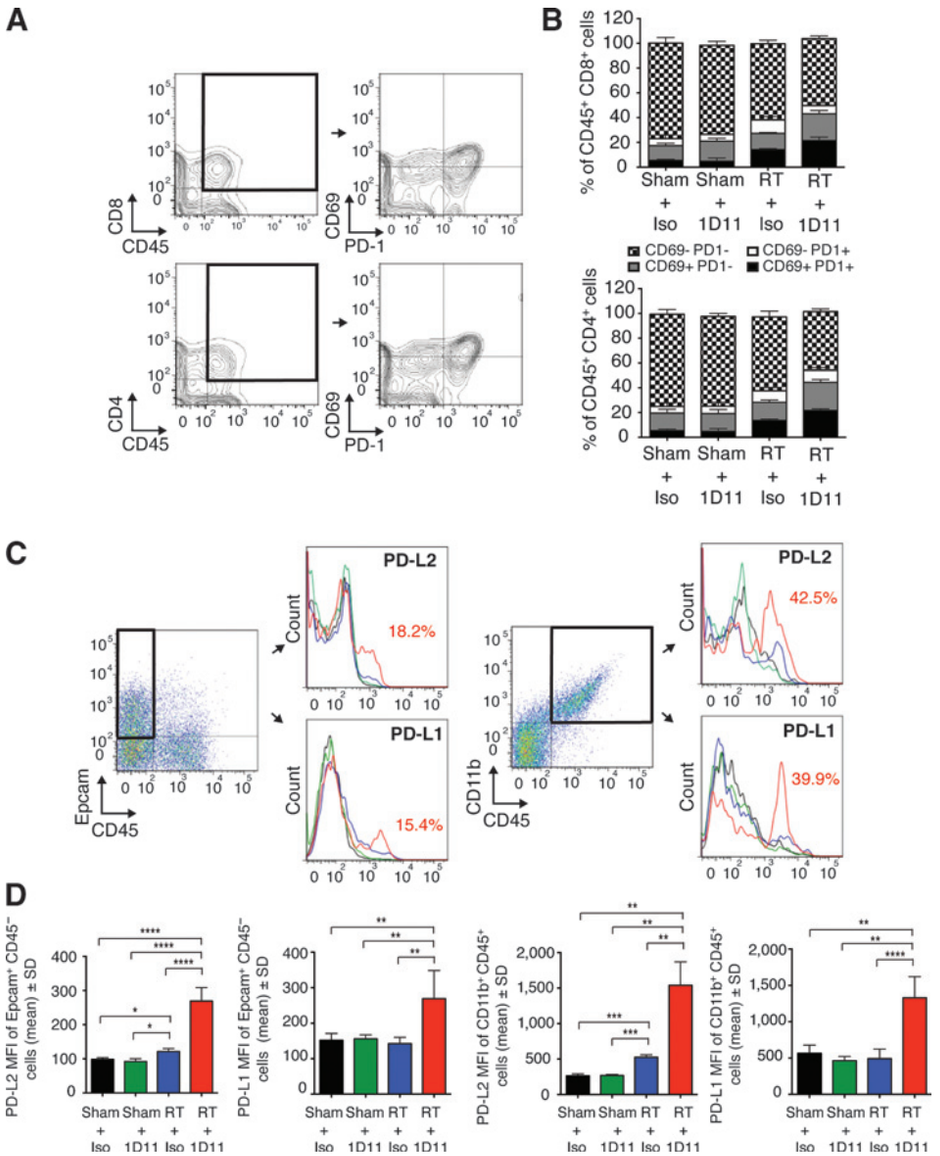

Figure 5.
Enhanced expression of PD-1 and its ligands in tumors treated with TGFβ blockade and RT. 4T1 tumors of mice treated as indicated in Fig. 1 were harvested at day 22 ($n = 9$/group). To obtain sufficient material, three tumors were pooled to obtain three independent samples for each group. A and B, expression of CD69 and PD-1 was analyzed in $CD4^+$ and $CD8^+$ T cells. Representative dot plots. B, bar graphs show mean percentage of T cells expressing one or both markers, as indicated. C, representative dot plots showing the gating strategy for 4T1 and myeloid cells, and expression of PD-1 ligands. D, bar graphs show PD-L2 and PD-L1 mean fluorescence intensity (MFI) ± SD on gated $Epcam^+$ $CD45^-$ 4T1 cells and $CD45^+CD11b^+$ myeloid cells. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$; ****, $P < 0.00005$.

exclude the contribution of other immune cells to the therapeutic effect achieved with RT and TGFβ blockade, depletion of T cells completely abrogated the inhibition of the primary irradiated tumor and nonirradiated lung metastases, demonstrating the critical role of T cells. When TGFβ was neutralized in irradiated tumors, we detected T cells reactive against multiple tumor antigens, including the antiapoptotic protein survivin and the transcription factor Twist, which promotes epithelial to mesenchymal transition and metastasis (48). Thus, TGFβ blockade is likely to be required to greatly enhance radiation-induced vaccination to breast and other cancers.

The prominent IFNγ signature and T-cell infiltration in tumors of mice treated with RT and TGFβ blockade are associated with development of adaptive immune resistance mediated by the PD-1 pathway (49). In fact, PD-L1 and PD-L2 were upregulated on both cancer cells and myeloid cells in tumors of mice treated with this combination. Increased expression of PD-1 was also seen in T cells infiltrating irradiated tumors, and was larger in the presence of TGFβ blockade. Administration of anti–PD-1 antibody enhanced tumor regression and delayed recurrence, demonstrating that PD-1 limits the function of T cells primed by RT and TGFβ blockade. Interestingly, tumor antigen-specific IFNγ production by $CD8^+$ T cells was enhanced by PD-1 blockade in the presence of RT, suggesting that improved priming of tumor-specific T cells may contribute to the therapeutic effect when PD-1 blockade is combined with RT-induced vaccination. Overall, data suggest a model whereby TGFβ controls priming of tumor-specific T cells by RT-released antigens, and PD-1 negatively modulates T-cell activation by acting at both the induction and effector phases of the antitumor response.

Despite the improved responses achieved with addition of anti–PD-1 to RT and TGFβ blockade, tumors still recurred within a relatively short time after discontinuation of anti–PD-1 treatment. It is possible that prolonged administration of anti–PD-1

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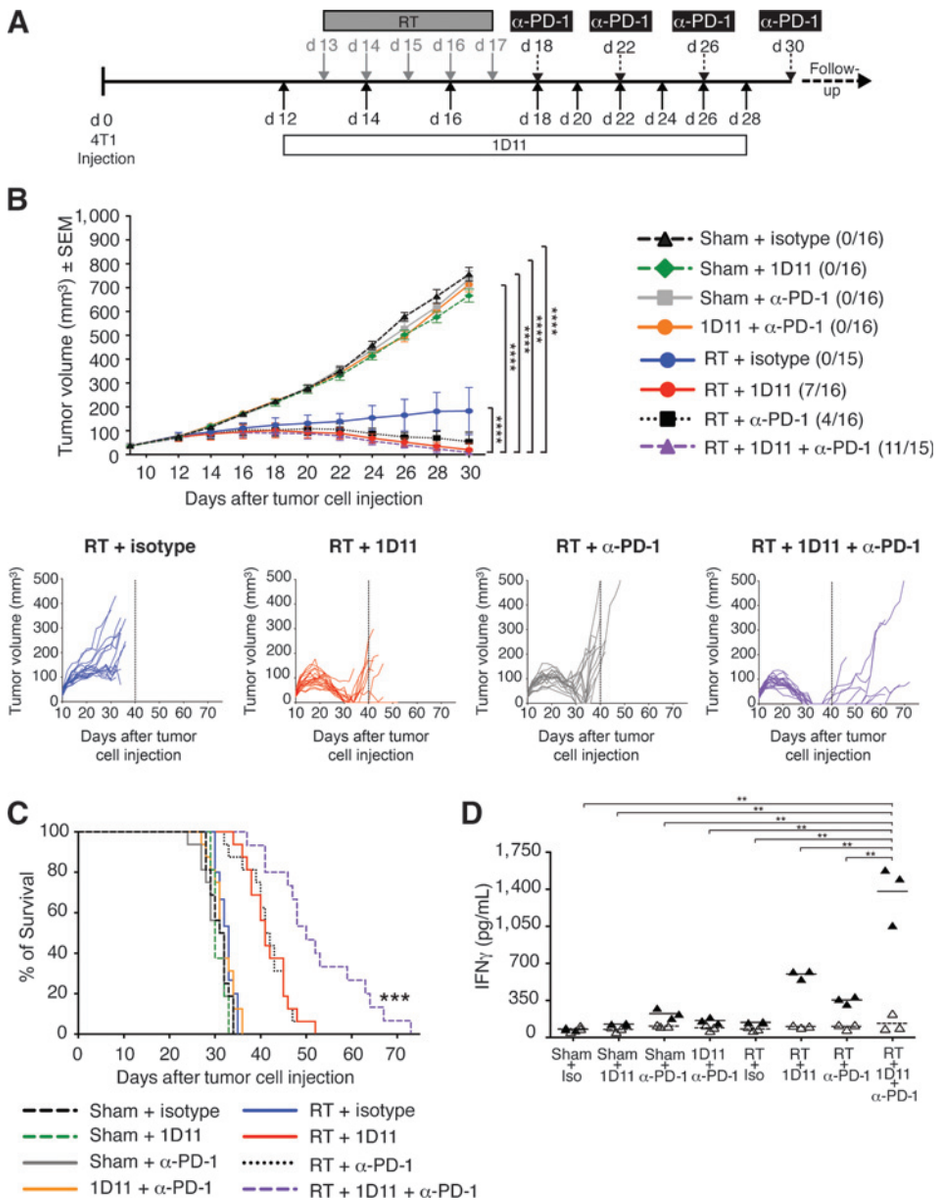

Figure 6.
Blocking PD-1 in mice treated with RT and TGFβ blockade improves tumor rejection and survival. A, treatment schema. B, mean tumor volume in each group up to day 30 and individual mice tumor growth curves for groups receiving RT. C, survival of mice treated with Sham+Isotype (dashed black line, $n = 16$), Sham+1D11 (dashed green line, $n = 16$), Sham+anti–PD-1 (gray line, $n = 16$), Sham+1D11+anti–PD-1 (orange line, $n = 16$), RT+Isotype (blue line, $n = 15$), RT+1D11 (red line, $n = 16$), RT+ anti–PD-1 (dotted black line, $n = 16$), and RT+1D11+anti–PD-1 (dashed purple line, $n = 15$). Data are representative of two independent experiments. D, IFNγ production by $CD8^+$ T cells from LN draining 4T1 tumors in response to AH1A5 peptide (closed triangles, $n = 3$) or irrelevant peptide ($n = 3$, open triangles). Each symbol represents one animal. Horizontal lines indicate the mean of antigen-specific (solid lines) or control (dashed lines) IFNγ concentration. Data are representative of two experiments. **, $P < 0.005$; ***, $P < 0.005$; ****, $P < 0.00005$.

would have further delayed or prevented recurrence. Alternatively, although T-cell responses to multiple tumor antigens were induced by RT and TGFβ blockade, it is possible that the critical antigenic target for tumor rejection is a tumor-specific mutant antigen (50) that might be lost allowing for tumor escape from immune control.

Overall, our findings support targeting of the PD-1 pathway in future clinical trials testing the combination of RT and TGFβ blockade, currently being tested in metastatic breast cancer patients (NCT01401062).

Disclosure of Potential Conflicts of Interest

M.H. Barcellos-Hoff reports receiving commercial research grant from Varian Medical Systems and has speakers' bureau honoraria from Genzyme and Isarna. S. Demaria is a consultant/advisory board member for Sanofi US Inc. and Regeneron Pharmaceuticals, Inc. S.C. Formenti is consultant/advisory board member for Regeneron, GSK, Sanofi. No potential conflicts of interest were disclosed by the other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Vanpouille-Box, J.M. Diamond, J. Zavadil
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Vanpouille-Box, J.M. Diamond, J. Zavadil, J.S. Babb, S. Demaria
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TGFβ Is a Master Regulator of Radiation Therapy-Induced Antitumor Immunity

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In situ vaccination by radiotherapy to improve responses to anti-CTLA-4 treatment

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ABSTRACT

Targeting immune checkpoint receptors has emerged as an effective strategy to induce immune-mediated cancer regression in the subset of patients who have significant pre-existing anti-tumor immunity. For the remainder, effective anti tumor responses may require vaccination. Radiotherapy, traditionally used to achieve local tumor control, has acquired a new role, that of a partner for immunotherapy. Ionizing radiation has pro-inflammatory effects that facilitate tumor rejection. Radiation alters the tumor to enhance the concentration of effector T cells via induction of chemokines, cytokines and adhesion molecules. In parallel, radiation can induce an immunogenic death of cancer cells, promoting cross-presentation of tumor-derived antigens by dendritic cells to T cells. Newly generated anti-tumor immune responses have been demonstrated post-radiation in both murine models and occasional patients, supporting the hypothesis that the irradiated tumor can become an in situ vaccine. It is in this role, that radiation can be applied to induce anti-tumor T cells in lymphocyte-poor tumors, and possibly benefit patients who would otherwise fail to respond to immune checkpoint inhibitors. This review summarizes preclinical and clinical data demonstrating that radiation acts in concert with antibodies targeting the immune checkpoint cytotoxic T-lymphocyte antigen-4 (CTLA-4), to induce therapeutically effective anti-tumor T cell responses in tumors otherwise non responsive to anti-CTLA-4 therapy.

1. Introduction

From the inception of carcinogenesis, the immune system detects and eliminates nascent tumors in a process described as cancer immunosurveillance. Stress-induced ligands and altered antigenicity render transformed cells susceptible to natural killers (NK) cells, γδ and conventional α/β T cells. Tissue disruption and unscheduled cell death that occur during tumor progression to invasion generate dangers signals in the form of damage-associated molecular pattern (DAMP) molecules that alert the immune system of a potential threat, activating both innate and adaptive immunity [1]. However, occasionally elimination of cancer cells is incomplete and cancer cells that have acquired the ability to evade immune control emerge, as a result of the selective pressure of the immune system. Thus, cancers rise to clinical detection after a long and complex crosstalk with the immune system, while a dominant immune suppressive tumor micro-environment has also emerged. The latter is enriched in cells with regulatory and immunosuppressive function that secrete cytokines such as transforming growth factor-β (TGFβ) and IL-10, which counteract immune-mediated rejection [2]. Noticeably, in some patients robust anti-tumor T cell responses are detectable at clinical diagnosis and their presence in the tumor specimen has been associated with a better prognosis [3,4]. Patients who retain such anti-tumor immunity appear to benefit the most from immunotherapy, even at advanced stages of the disease [5]. For example, responses to immune checkpoint inhibitors rely on the patient's pre-existing anti-tumor T cells [6,7]. Unfortunately, only a small fraction of cancer patients retains sufficient anti-tumor immune responses. Among solid tumors patients, melanoma carriers are most likely to respond to immune checkpoint inhibitors targeting CTLA-4 or programmed cell death-1 (PD-1) [8,9], possibly because of their high mutational load [10].

Because responses to anti-CTLA-4 often are durable [11,12], identifying combination treatments that can convert patients

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unresponsive to CTLA-4 inhibition into responders is an active area of investigation. Potential candidates include other immunotherapies, standard chemotherapy, targeted agents [13–15], and radiotherapy has earned a prominent place, due to substantial preclinical data [16–20] and rapidly accumulating clinical observations [21–23] that it can induce therapeutically effective anti-tumor immunity when combined with CTLA-4 blockade. Several clinical trials are currently ongoing to test radiotherapy in combination with the FDA-approved anti-CTLA-4 antibody ipilimumab (Yervoy®, Bristol Meyers-Squibb, New York, New York) (Table 1).

Here we review the available data that has informed the rationale for exploiting the synergy of radiation and CTLA-4 blockade.

2. Radiation-induced in situ tumor vaccination

Over the past decade, an improved understanding of the effects of local radiation on tumor-host interactions has led to the recognition that radiotherapy may have a novel role as an inducer of acute inflammation and immunogenic cell death, capable to convert a tumor into an in situ vaccine [24–26]. Pioneering work implicating T cells in determining the response to radiation was published several decades ago [27]. More recently, the demonstration that T cells mediate the abscopal effect (out-of-field responses) of radiation in a pre-clinical tumor model [28] has provided a putative mechanism for the intriguing clinical observation that rare patients with disseminated cancer experienced systemic tumor regression after irradiation of a single tumor site [29–32].

2.1. Radiation induces an immunogenic death of cancer cells and priming of tumor-specific T cells

Multiple mechanisms that contribute to radiation-induced anti-tumor immunity are emerging and the signals generated by irradiated dying tumor cells are being elucidated.

Priming of anti-tumor immune responses by cytotoxic treatments has been shown to require the presence of an immunogenic cell death (ICD) [33]. ICD relies on the orchestration of specific molecular signals that stimulate cross-presentation of tumor cell antigens by dendritic cells (DCs) to T cells [34]. Mediated by endoplasmic reticulum (ER) stress and autophagy, ICD is characterized by cell surface translocation of calreticulin (CRT), and extracellular release of high-mobility group protein B1 (HMGB1) and ATP [35–37]. CRT is an ER-associated chaperone protein that when expressed on the surface of dying cells promotes their phagocytosis acting as an “eat-me” signal for DCs [38]. HMGB1 is a nuclear protein found in almost all mammalian cells and is secreted by a variety of immune cells to induce pro-inflammatory effects when bound to pattern-recognition receptors (PRRs) such as the toll-like receptor-4 (TLR4) [39]. During cell death extracellular release of HMGB1 delivers both chemotactic and maturation signals to DCs, promoting phagocytosis of dying tumor cell and DC migration to lymph nodes (LN) to cross-present antigens and prime T cells [40,41]. ATP released from dying cells binds to P2X7 purigenic receptor on DCs and activates the inflammasome, leading to secretion of interleukin (IL)-1β [37]. While in vivo the relative contribution of each of these factors to priming of anti-tumor T cells remains undefined, radiation has been shown in vitro to generate all three ICD molecular signals [42–44].

Recently, additional mechanisms that play a critical role in radiation-induced anti-tumor T cell priming have been described. Gupta et al. showed that a single 10 Gy radiation dose induced the activation of intratumoral DCs, measured as up-regulation of co-stimulatory molecules CD86 and CD70 [45]. Activation of intratumoral DCs was shown by Burnette et al. to first require their production of type I interferon (IFN-I) and to be critical for radiation-induced T cell cross-priming [46]. Moreover, Deng et al. demonstrated that tumor-derived DNA induced IFN-I production by DCs via the stimulator of IFN genes (STING) pathway [47].

It is important to notice that the experimental settings demonstrating priming of T cells by radiation consist of relatively

Table 1
Ongoing clinical trials testing the combination of CTLA-4 blockade and radiation therapy (RT).

Identifier	Condition	Intervention	Phase
NCT01689974	Metastatic melanoma	Ipilimumab Radiation Therapy and Ipilimumab	Phase 2
NCT02254772	B-cell Lymphomas, multiple types	Ipilimumab TLR9 agonist SD-101 Radiation therapy	Phase 1 Phase 2
NCT01703507	Recurrent Melanoma Stage IV Melanoma Tumors Metastatic to Brain	Ipilimumab Whole-Brain Radiation Therapy (WBRT) Stereotactic Radiosurgery (SRS)	Phase 1
NCT02239900	Liver cancer Lung cancer	Ipilimumab Stereotactic Body Radiation Therapy (SBRT)	Phase 1 Phase 2
NCT01970527	Recurrent melanoma Stage IV Melanoma	SBRT Ipilimumab	Phase 2
NCT01557114	Malignant melanoma	Ipilimumab Radiotherapy	Phase 1
NCT02221739	Non-small cell lung cancer	Ipilimumab Intensity-modulated radiation therapy (IMRT)	Phase 2
NCT01449279	Melanoma	Ipilimumab Radiation Therapy	PILOT
NCT01565837	Melanoma	Ipilimumab Stereotactic Ablative Radiosurgery (SART)	Phase 2
NCT02107755	Liver Metastases Lung metastases Recurrent melanoma Stage IV melanoma Tumors metastatic to brain	Iipilimumab Stereotactic radiosurgery	Phase 2
NCT01996202	High Risk Melanoma	Ipilumimab Radiation	Phase 1
NCT02097732	Metastatic melanoma Brain metastases	Ipilimumab Stereotactic Radiosurgery (SRS)	Phase 2
NCT01950195	Newly diagnosed melanoma metastases in the brain and spine	SRS Ipilimumab	Phase 1
NCT01711515	Cervical Carcinoma	Cisplatin External beam radiation therapy Brachytherapy Ipilimumab	Phase 1
NCT01935921	Stages III and IV head and neck cancer	Cetuximab IMRT Ipilimumab	Phase 1
NCT02115139	Melanoma Brain metastases	Ipilimumab WBRT	Phase 2
NCT01860430	Head and Neck cancer	Cetuximab/IMRT Plus Ipilimumab	Phase 1
NCT01730157	Ocular Melanoma Extraocular extension melanoma Metastatic ocular melanoma	Ipilimumab yttrium Y 90 glass microspheres	Phase 0
NCT02406183	Metastatic melanoma	Ipilimumab SBRT	Phase 1

immunogenic mouse tumors and/or used strong model antigens such as ovalbumin (OVA) as reporter antigens [48,49]. In the setting of poorly immunogenic murine tumors, that better model the clinical reality of cancer, radiation by itself is generally insufficient to prime T cell responses [50,51].

One explanation for the suboptimal vaccination induced by radiation it the possible concomitant activation of immunosuppressive signals. Our recent data support this hypothesis. In two poorly immunogenic murine carcinomas blockade of TGFβ, which is activated from its latent form by reactive oxygen species (ROS) generated by radiation [52], was required for priming of CD8 T cells specific for three endogenous antigens: The anti-apoptotic protein survivin, the transcription factor twist-1 and, the gp70 envelope glycoprotein of an endogenous retrovirus [53].

Another mechanism of radiation-induced immunosuppression is mediated by the conversion of ATP by CD39 and CD73 into adenosine, which suppresses anti-tumor T cell activation, survival and effector function via the A2a adenosine receptor (A2AR). Adenosine also negatively modulates differentiation and function of DCs and natural killer (NK) cells [54,55]. Thus, at least in some tumors adenosinergic pathways may limit the ability of radiation to induce effective anti-tumor immunity.

Other mechanisms that have been suggested to hamper radiation-induced immunization are a relative increase in regulatory T cells (Tregs) and in immunosuppressive myeloid cells post-radiation [56,57].

Information about the breath and specificity of the anti-tumor T cell responses primed by radiation is very limited. The antigenic repertoire of a given tumor classically includes self-antigens, like overexpressed differentiation antigens and Cancer–Testis (CT) antigens [58]. Although T cells can recognize them, some degree of T cell tolerance usually exists as the result of elimination of T cells with high affinity T cell receptor (TCR) during T cell ontogeny. In experimental models, radiation fails to induce T cell responses to these relatively weak antigens [53]. However, increase in T cell responses to self-antigens such as survivin could be detected in occasional patients undergoing radiotherapy [51]. Interestingly, it has been shown that radiation induces the expression of novel epitopes derived from protein that are transcribed in response to radiation-induced damage of cancer cells [59]. Nevertheless it is unclear whether T cells specific for radiation-induced antigens are elicited that contribute to the rejection of the irradiated tumor. Finally, a class of tumor antigens that can elicit strong T cell responses are mutated neo-antigens [60]. Recent evidence suggests that such mutated neo-antigens are the targets of T cells during successful tumor rejection and our group is actively investigating whether radiation can induce such responses.

2.2. Radiation promotes a pro-immunogenic tumor microenvironment

In addition to the signals released by dying cells after exposure to ionizing radiation, signals released by tumor and stromal cells that survive radiation damage modulate the tumor microenvironment [61,62]. Effects of radiation that facilitate the effector phase of anti-tumor immune responses include upregulation of specific chemokines and cell surface receptors, as well as vascular changes.

Cancer cells are the source of some of the chemokines induced by radiation. For example, in vitro, mouse and human breast cancer cells, and mouse prostate, colon carcinoma and fibrosarcoma cells increased levels of CXC chemokine ligand (CXCL) 16 upon radiation exposure to doses in the range of 2 to 12 Gy [19,63]. In vivo, CXCL16 was critical for efficient recruitment of effector CD8 T cells to irradiated mouse 4T1 breast tumors [19]. In the B16-OVA mouse melanoma model, after radiation the cancer cells produced CXCL9 and CXCL10 in response to IFN-γ produced by infiltrating hematopoietic cells [64].

Radiation has also been shown to promote normalization of aberrant vasculature and activation of endothelial cells with upregulation of vascular adhesion molecules, leading to improved tumor infiltration by T cells [48,65]. Interestingly, in a mouse model of pancreatic cancer vascular normalization was achieved with a single 2 Gy dose of radiation, improving tumor rejection by adoptively transferred T cells [65].

Finally, a number of phenotypic changes defined by some investigators as “immunogenic modulation” have been described to occur in neoplastic cells surviving radiation exposure [62]. For instance, post-radiation upregulation of Major Histocompatibility Complex (MHC) class I molecules and Fas death receptor on tumor cells promoted tumor rejection by CD8+ T cells in vivo [59,66,67]. Moreover, RT induces expression of natural-killer group 2, member D (NKG2D)-ligands, powerful stimulators of both NK and CD8+ T cells [68].

2.3. Role of radiation dose and fractionation

Many of the pro-immunogenic effects of radiation on the cancer cells described above can be induced, at least in vitro, by radiation doses varying from about 2 Gy to as much as 30 Gy or more, but the optimal radiation regimen to induce clinical anti-tumor immunity remains to be defined. Only a few studies have compared different radiation doses and fractionation, or conducted the same experiments in tumor cells with different intrinsic radiosensitivity, [69]. In vitro, a dose-dependent increase of the ICD signals and of some chemokines and surface molecules were reported [19,43,59]. In vivo, the nature of the pre-existing tumor microenvironment at the time of radiation and the response of normal stromal cells present within the field of radiation are important determinants of the development of effective anti-tumor immunity. For instance, Klug et al overcame the immunosuppressive effect of hypoxia by a low dose single dose radiation (2–5 Gy) that resulted in re-programming of tumor-infiltrating macrophages [65]. On the other hand, priming of OVA-specific T cells was shown to be more pronounced when B16-OVA tumors were irradiated with 15 Gy given as single dose rather than as 5 fractions of 3 Gy each [48]. In another study using the same tumor model, Schaue et al. reported that while a single dose of 15 Gy primed OVA-specific T cells, it also led to a relative increase in Tregs, and showed that the best ratio of anti-tumor T cells to Tregs was achieved when radiation was given in two fractions of 7.5 Gy [70]. These examples highlight the complex interaction between the irradiated tumor and the host immune system, and suggest that studies to optimize the radiation regimen need to take into consideration multiple parameters, likely to also be tumor-type specific. Moreover, combinations of radiation with different immunotherapy strategies may require specific dose regimens and fractionation.

Finally, while radiation can convert the tumor into an in situ vaccine, its effects on the tumor microenvironment persist and evolve long after the time of radiation exposure. The degree and duration of these effects may depend on the degree of pre-existing immunosuppression and the balance between signals that promote tumor rejection versus signals that hinder it. In most cases, radiation therapy alone is unable to induce effective immune-mediated tumor rejection: however it may potentiate immunotherapy, as exemplified by preclinical and clinical work with immune checkpoint blocking agents. We will discuss below the combination of radiation with anti-CTLA-4 antibodies.

3. Cytotoxic T lymphocyte antigen-4, a negative regulator of T-cell activation

An array of co-stimulatory and co-inhibitory molecules regulates T cells activation, balancing the need to eliminate pathogens with the prevention of autoimmunity [71]. T cell activation requires two signals, the first is delivered by TCR binding to MHC-I/antigen. The second is delivered by CD28 costimulatory receptor that binds to CD80 (B7–1) and CD86 (B7–2) on the surface of antigen presenting cells (APC), resulting in abundant secretion of IL-2 and T-cell proliferation [72].

After TCR engagement CTLA-4 is rapidly recruited to the immune synapse where it competes with CD28 for binding to CD80 and CD86. Because of its greater affinity, when co-stimulation is suboptimal interactions between CTLA-4 and costimulatory molecules prevail, thus impeding T cell proliferation [73]. Importantly, CTLA-4 not only hinders the interaction between CD28 and CD80/86 but also impairs T cell activation by dephosphorylating key effector molecules required for TCR signaling [74,75]. Additionally, when constitutively expressed on Tregs, CTLA-4 has been shown to reduce expression of CD80 and CD86 on APCs as well as to increase the secretion of TGFβ, thus fostering T cell tolerance to tumors by multiple mechanisms [76,77].

3.1. Effects of anti-CTLA-4 antibodies on priming of anti-tumor T cells

The role of CTLA-4 in limiting the development of anti-tumor immune responses in cancer patients has been demonstrated by the therapeutic success of anti-CTLA-4 monoclonal antibody (mAb) in metastatic melanoma [9]. The mechanisms by which anti-CTLA-4 mAbs unleash anti-tumor immunity remain incompletely understood. Preclinical studies have shown that CTLA-4 blockade enhances anti-tumor immunity by reducing the threshold for T cell activation [78], resulting in monoclonal and oligoclonal expansion of CD4 T cells responding to cognate peptide antigens or superantigens [79]. Importantly, work by Jim Allison's lab demonstrated that CTLA-4 plays a major role in shaping the breadth of reactivity of a primed T cell population, by constraining the "best-fit" population [80]. Thus, when CTLA-4 is blocked, the lower threshold required for T cell activation allows proliferation and expansion of tumor antigen-specific T cells, leading to tumor rejection. Importantly, Kvistborg et al. have recently provided evidence for a similar broadening of melanoma-reactive T cell responses in patients treated with anti-CTLA-4 [81].

3.2. Effects of anti-CTLA-4 antibodies on the effector phase of tumor rejection

The ability of anti-CTLA-4 mAb to promote anti-tumor immunity is not only confined to the priming phase. A common feature associated with anti-CTLA-4 mediated tumor rejection is an increase in the ratio of effector T to Tregs cells within the tumor [82,83]. While effector T cells are increased due to the pro-proliferative effect of CTLA-4 blockade, recent preclinical data demonstrated that depletion of intratumoral Tregs also plays a major role in tumor rejection. Anti-CTLA-4 mAbs capable of mediating antibody-dependent cellular cytotoxicity (ADCC) effectively and selectively eliminated intratumoral Tregs, which express higher levels of CTLA-4 compared to circulating Tregs [84–86]. CTLA-4 ligation on effector T cells has also been shown to enhance motility of both CD4 and CD8 T cells [20,87,88]. Using CD8 T cells expressing a Pmel-1 transgenic TCR specific for a melanoma antigen Pentcheva-Hoang et al. showed that increased motility was associated with tumor rejection, and hypothesized that anti-CTLA-4 may act by reversing motility paralysis of exhausted intratumoral T cells [87].

4. Synergy of radiotherapy with anti-CTLA-4 antibody

Previous work in pre-clinical models of melanoma and breast cancer showed that tumors insensitive to anti-CTLA-4 treatment as monotherapy became responsive upon vaccination with modified autologous tumor cells [89,90]. We hypothesized that in situ vaccination by radiation could also convert a poorly immunogenic tumor, unresponsive to anti-CTLA-4 into a responder. This hypothesis was confirmed in three different murine tumor models, 4T1 and TSA mammary carcinomas syngeneic to BALB/c mice and MCA38 colorectal carcinoma syngeneic to C57BL/6 mice [16,17]. Importantly, the anti-tumor T cells elicited by the combination not only did reject the irradiated tumor but also inhibited spontaneous 4T1 lung metastases or synchronous unirradiated tumors (abscopal effect) in mice bearing TSA and MCA38 carcinomas [16,17].

The mechanisms of synergy between radiation and anti-CTLA-4 mAb were studied in more details in the 4T1 model. These studies showed that anti-CTLA-4 therapy and radiation interact at multiple levels, each contributing to tumor rejection (Fig. 1). First, development of CD8 T cell responses to the tumor was required for tumor rejection [16,18]. Priming of CD8 T cells was monitored in tumor-draining lymph nodes by measuring IFNγ production to the AH1 gp70-derived CD8 T cell epitope. Radiation and anti-CTLA-4 antibody induced measurable T cell priming only when used in combination, indicating that each treatment provided critical non redundant signals. The number of DCs available locally to cross-present the antigens released by radiation determined the magnitude of the elicited anti-tumor T cell responses [91]. We found that in 4T1 tumor-bearing mice the number of DCs present within the tumor and draining lymph nodes was regulated by invariant natural killer T (iNKT) cells. Blocking CD1d-mediated interaction of iNKT cells with DCs led to an increase in DCs and improved CD8 T cell priming and overall tumor response to the combination of radiation therapy and anti-CTLA-4 treatment [91].

In addition, radiation-induced molecular signals were required for tumor rejection. For instance, CXCL16 upregulation by radiation was required for effector CD8 T cell homing to 4T1 tumors. CXC chemokine receptor (CXCR) 6-deficient mice, whose T-cells are unable to respond to this chemokine, failed to show increased CD8 T cells infiltration post-treatment and response to radiation and anti-CTLA-4 [19].

Finally, intravital imaging using two photon laser scanning microscopy was used to study in vivo how radiation affected the interaction of CD8 T cells with 4T1 cancer cells. To track the polyclonal population of CD8 T cells activated by treatment with radiation and anti-CTLA-4 we used $CXCR6^{+/gfp}$ mice. In these mice, GFP expression within the tumor was largely confined to activated tumor-specific CD8 T cells, as determined by their expression of activation markers, ex vivo IFNγ production and by the fact that blocking MHC-I disrupted the interaction of GFP^+ T cells with CFP^+ 4T1 cells [20]. Some activated CD8 T cells were present in all tumors, although over time they only increased in tumors of mice treated with radiation and anti-CTLA-4. Motility of these T cells was mildly enhanced in irradiated tumors and markedly enhanced in tumor of mice treated with anti-CTLA-4 monotherapy. In contrast, when these two treatments were combined, activated CD8 T cells displayed reduced motility, forming stable contacts with 4T1 cells. Interactions between NKG2D receptor and its ligand retinoic acid early inducible-1 (RAE-1), which was induced by radiation on 4T1 cells, were required to achieve the stable contacts between tumor and CD8 T cells. Blocking NKG2D with an antibody increased CD8 T cell speed and abrogated the tumor inhibition achieved with

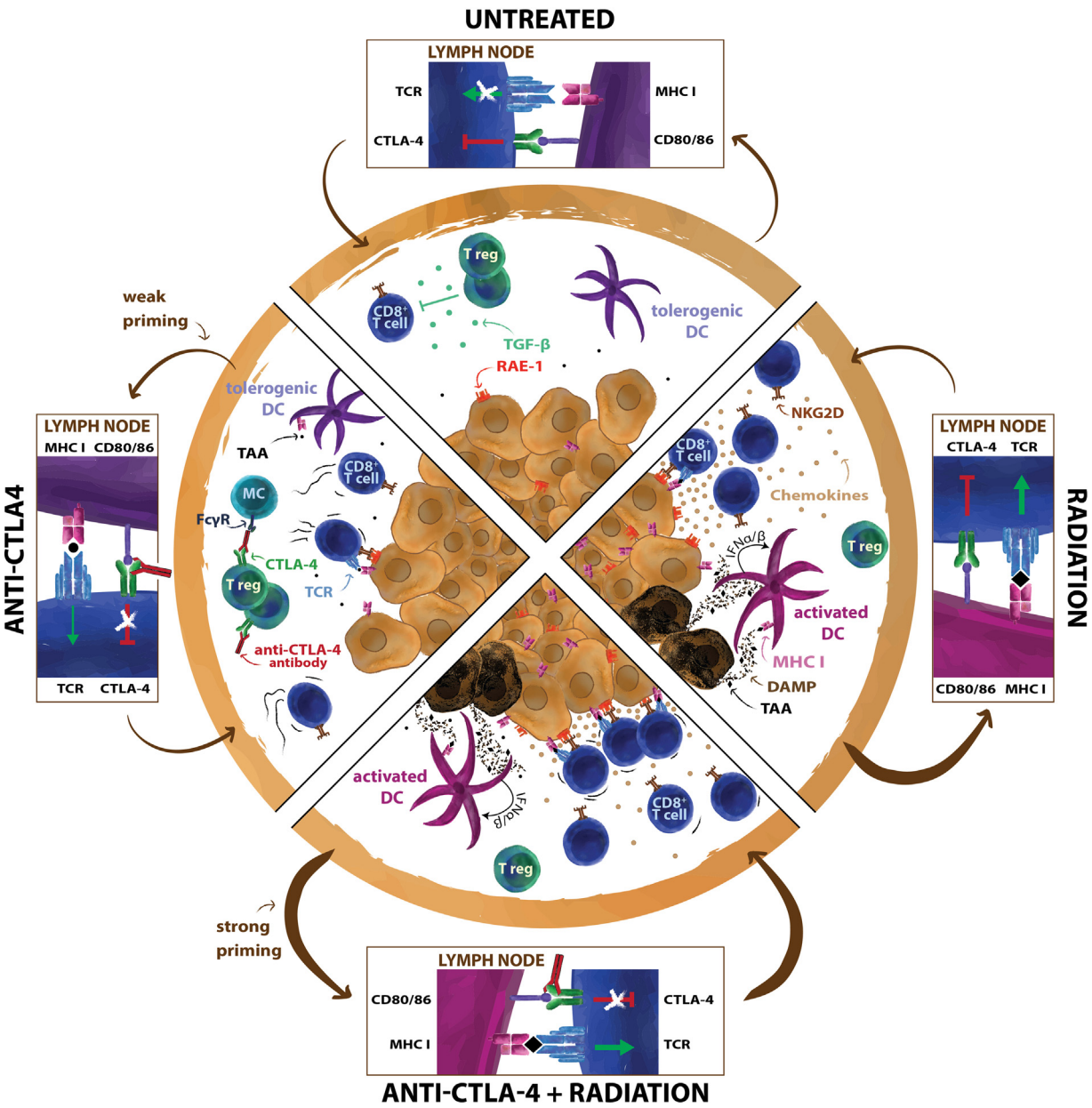

Fig. 1. *Mechanisms of synergy between radiotherapy and anti-CTLA-4 treatment.* Schematic illustration highlighting the critical changes induced by each treatment in the tumor-draining lymph nodes (dLNs) and tumor. Untreated tumors: priming of T cells in dLN is limited by negative signals delivered by CTLA-4. In the tumor, tumor rejection by CD8 T cells is hampered by low MHC class I and immune-stimulatory ligands (e.g., RAE-1) on tumor cells, and by the immunosuppressive tumor microenvironment, rich in TGFβ, Tregs and tolerogenic DCs. Anti-CTLA-4 treatment: activation and expansion of tumor-specific T cells in dLNs is improved by blocking CTLA-4-mediated negative signal, but it remains suboptimal in the setting of poorly immunogenic tumors due to low antigen availability and low activation of DC. Clearance of intratumoral CTLA-4^{hi} Tregs is mediated by anti-CTLA-4 mAb via ADCC if FcγR-expressing myeloid cells are present. At the same time, the pro-motility effects of CTLA-4 ligation hinder the formation of a stable immune synapse between activated CD8 T cells and tumor cells. Radiation: induction of ICD provides tumor antigens and activation signals to DCs, but cross-presentation of tumor-derived antigens to CD8 T cells in dLNs is limited by inhibitory signaling via CTLA-4. Radiation combined with anti-CTLA-4: priming of tumor-specific CD8 T cells is markedly enhanced by complementary effects of radiation and anti-CTLA-4. In addition, activated T cell homing to the tumor is facilitated by increased levels of chemokines (CXCL16 and CXCL10) released by tumor cells in response to radiation. Inside the tumor, co-engagement of TCR and NKG2D on CD8 T cells by radiation-induced MHC-I and RAE-1 allow formation of stable immune synapses with tumor cells. Overall, radiation and anti-CTLA-4 therapy have complementary effects that underlie their synergistic interaction in inducing tumor rejection.

radiation and anti-CTLA-4 [20]. Thus, in the presence of anti-CTLA-4 antibody radiation is providing a signal to promote the formation of an effector immune synapse between CD8 T and tumor cells. The requirement for co-engagement of TCR and NKG2D to achieve a stop signal when CTLA-4 is ligated was supported by additional in vitro data [20]. Intriguingly, while a similar motility enhancement by anti-CTLA-4 treatment was recently reported in intratumoral CD8 T cells expressing a transgenic TCR specific for a melanoma antigen, in the latter experimental system enhanced motility resulted in more effective tumor rejection [87]. This difference may reflect the different requirements for formation of an effector immune synapse for a homogeneous T cell population expressing a TCR with relatively high affinity for an abundant tumor antigen. In our experimental system we studied a polyclonal T cell population with TCRs of varying affinity for antigens that may be expressed at very low levels within the tumor. Low affinity/avidity

TCR interactions with tumor cells will be dependent on NKG2D co-engagement and killing may require a more protracted interaction [92].

Overall, this data supports the multiplicity of effects of radiotherapy on the efficacy of immunotherapy targeting the checkpoint receptor CTLA-4. It is likely that dominant mechanism(s) of interaction between these two modalities mainly depend on the pre-existing tumor microenvironment and host general immune status.

However the type of radiation regimen used may also be a determinant of success. In a comparison of two fractionated radiation regimens (8 Gy × 3 and 6 Gy × 5) and a single large dose (20 Gy) in two tumor models, TSA and MCA38, we found that effective anti-tumor immunity leading to rejection of the irradiated and non-irradiated synchronous tumors (abscopal effect) was only achieved by the fractionated regimens, while the single 20 Gy dose failed to show synergy with anti-CTLA-4 [17]. While the reasons for this difference are being actively investigated, it is intriguing that the two most notable cases of abscopal responses seen in patients with melanoma and lung cancer treated with radiotherapy and ipilimumab occurred with the use of similar fractionated radiotherapy regimens, 9.5 Gy × 3 in the melanoma case, and as 6 Gy × 5 in the lung cancer case [21,23].

5. Clinical translation

Since 2011, after the approval of ipilimumab for patients with metastatic or unresectable melanoma, a few dramatic abscopal responses have been reported after radiation of one metastasis in patients who were unresponsive or had ceased to respond to ipilimumab [21,22,93]. These reports have sparked several retrospective analyses of outcome in melanoma patients receiving radiation while treated with ipilimumab, with an excellent review of these studies recently published by Barker and Postow [94]. Most of the patients in these retrospective series received radiation to the brain, and in most cases the analysis suggested a survival benefit associated with the combination. A retrospective study in a cohort of 21 patients who received radiation to the brain or extracranial sites after progression on ipilimumab reported abscopal responses in 62%, which were associated with increased survival [95]. While these cases seem to confirm the preclinical synergy of radiation with ipilimumab, a caveat is the fact that since melanoma is known to respond to ipilimumab the findings could represent late responses attributable to ipilimumab alone. Several prospective trials are ongoing to determine the benefits of the combination of radiation with anti-CTLA-4 antibodies in melanoma (Table 1).

Results of a phase I study in 22 melanoma patients with escalation of the radiation dose were recently reported and confirmed that radiation did not worsen the toxicity expected with ipilimumab alone. There were no complete responses and overall disease control rate was not much higher than what would be expected with ipilimumab alone in melanoma [96]. While this study does not provide conclusive evidence about the benefits of radiation combined with ipilimumab it raises the question of whether two doses of either 6 or 8 Gy, which were received in 12 out of 22 patients, may be suboptimal in melanoma to achieve in situ vaccination.

A large randomized trial in metastatic castrate-resistant and docetaxel-refractory prostate cancer compared radiation given as a single 8 Gy dose to a bone metastasis with radiation plus ipilimumab. Overall, there was no significant difference in survival between the two arms, but a benefit in the combination arm was seen among patients with good prognostic features [97]. This data suggests that the degree of immune competence may influence the likelihood of response, and again raises the question whether a single 8 Gy dose, may explain the limited success of this study [17]. We have reported a complete abscopal response in a patient with non small cell lung cancer treated with radiation given in 5 fractions of 6 Gy each and ipilimumab [23]. Since lung cancer has been shown to be insensitive to anti-CTLA-4 monotherapy [98] this case supports the hypothesis that radiation could be used to sensitize unresponsive tumor types to anti-CTLA-4 treatment. This hypothesis is currently being tested in a clinical trial (NCT02221739).

6. Conclusions

The ability of radiation to elicit anti-tumor immune responses has been unequivocally demonstrated in experimental models, and many of the mechanisms involved have been identified. However, more work is required to define the dose(s) and fractionation that optimally induce anti-tumor T cells, and identify the tumor characteristics that predict which tumors will respond to a given combination of radiation and immune checkpoint blockade. While the growing number of reports of occasional abscopal responses in patients receiving radiation therapy and anti-CTLA-4 antibody has generated a lot of interest, the results of the first two completed prospective trials testing radiation and anti-CTLA-4 highlight the need to carefully design future studies. Recent data have emphasized the importance of T cell responses to unique individual tumor antigens [60]. In this context, local radiation deserves to be thoroughly explored for its potential to offer an attractive, easy to use and cost-effective intervention for personalized tumor vaccination.

Conflict of interest statement

The authors declare that no conflict of interest exists.

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POSTER PRESENTATION – 2015 AACR annual meeting.

Abstract 2493: Fractionated but not single dose radiation is an optimal adjuvant for in situ tumor vaccination.

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Local radiotherapy (RT) has the potential to generate an in situ tumor vaccine by inducing an immunogenic cell death (ICD) of the cancer cells. In vitro, generation of the signals that define ICD is radiation dose-dependent. However, in vivo we have previously shown that radiation doses of 8 and 6 Gy given in 3 to 5 consecutive days were more effective at inducing anti-tumor T cell responses in combination with anti-CTLA-4 antibody than a single larger dose of 20 Gy (Dewan et al., Clin Cancer Res 2009).
To understand the mechanisms underlying the different outcome obtained with fractionated versus single dose RT, TSA tumors growing in syngeneic immunocompetent BALB/c mice were harvested at 4, 24 and 48 hrs post irradiation with 8 Gy X 3 or 20 Gy X 1 for analysis of gene expression using Agilent mouse mRNA Microarray Kit (V2). Subsequent data were analyzed using Ingenuity Pathway Analysis (IPA) and gene ontology classification. Expression of key immune genes in TSA cells irradiated in vitro was assessed by RT-qPCR. Tumor-infiltrating dendritic cells (TIDC) were analyzed 5 days after the last radiation exposure by flow cytometry. IFNβ production by irradiated TSA cells was measured by Procarta™ immunoassay.

Over 100 immune response genes were differentially expressed in tumors irradiated with 8Gyx3 but not 20Gyx1, with a dominant type I interferon (IFN) response at 4 and 24 hours, which was confirmed by RT-qPCR. Tumor infiltration by CD8α+ dendritic cells (DC), which are the subset of DC cross-presenting tumor cell-derived antigens, was only slightly increased by 20Gyx1 but markedly increased by 8Gyx3. Moreover, DCs present in tumors treated with 8Gyx3 showed a significant upregulation of activation markers CD40 and CD70. Importantly, the in vitro setting (devoid of an immune infiltrate) demonstrated expression of IFNβ and downstream immune genes, including chemokines CXCL9, CXCL10 and CXCL11 by TSA cells irradiated with 8Gyx3 but not 20Gyx1. Production of IFNβ by TSA cells was confirmed with an increased secretion of IFNβ in the supernatant only after 8Gyx3 irradiation.

Overall, data indicate that fractionated RT can mimic, at least in part, a viral infection and activate canonical defense pathways in neoplastic epithelial cells with induction of type-I IFN. In vivo this leads to recruitment and activation of DC cross-presenting tumor antigens. Thus, fractionated-RT acts as an optimal immune adjuvant for in situ tumor vaccination. We propose that repeated cycles of RT-induced cell death may better mimick cycles of viral infection and are currently defining the molecular mechanisms involved. Results are important for understanding the determinant of response in patients currently treated wit RT and anti-CTLA-4 in clinical trials at our institution.

POSTER PRESENTATION – 2015 CRI-CIMT-EATI-AACR

Abstract A144: Cooperative effects of TGFβ and activin A control regulatory T cells numbers in irradiated tumors.

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Transforming Growth Factor-beta (TGFβ) and activin A (actA) are members of the TGFβ superfamily. TGFβ and actA display overlapping biological activities including the ability to promote the conversion of conventional CD4 T cells to regulatory T cells (Tregs). We have recently shown that in situ vaccination by local tumor irradiation is hindered by activation of latent TGFβ (Vanpouille-Box et al., Cancer Res 2015). Intriguingly, while TGFβ blockade enhanced activation of dendritic cells (DC) and T cell priming, it did increase rather than reduce intratumoral Tregs. Because there is evidence that actA and TGFβ pathway cross-regulate each other, we tested the hypothesis that upregulation of actA by RT in the presence of TGFβ blockade may be responsible for the observed Tregs increase within the tumor.

Secretion of actA by untreated and irradiated 4T1 tumor cells was quantified by ELISA. Transwell co-culture was used to assess the ability of actA released by irradiated cancer cells to promote conversion of naïve CD4 T cells into Tregs. 4T1 derivatives with conditional actA knockdown ($4T1^{shActA}$) or non-silencing control ($4T1^{shNS}$) were engineered using a set of inducible tetracycline plasmids and injected s.c. in BALB/c mice (day 0). *ActA* gene knockdown was induced by doxycyline at day 8. TGFb neutralizing 1D11 or isotype control antibodies were given i.p. every other day starting on day 12. RT was delivered to the primary tumor in 6Gy fractions on five consecutive days beginning on day 13. Mice were followed for tumor growth or euthanized at day 22 to harvest tumor draining lumph node (TDLN) for ex vivo restimulation and tumors to evaluate Tregs infiltration.

RT significantly increased actA secretion (4T1: 278.8 pg/mL for 10^5 cells/24h; irradiated 4T1: 892.2 pg/mL for 10^5 cells/24h; $p<0.05$). Conversion of naïve CD4+ T cells into Treg upon activation in the presence of irradiated 4T1 cells was markedly enhanced (Control: 8.1%, irradiated 4T1: 46.9% of Treg). This effect was partially reversed in the presence the actA inhibitor follistatin (23.8% of Treg). As expected, *ActA* gene expression was upregulated in irradiated 4T1-tumors but was markedly increased in mice treated with 1D11 and RT+1D11. Neither in vivo TGFβ blockade nor *ActA* gene knockdown by themselves affected tumor growth. However, each intervention significantly improved tumor control achieved by RT. TGFβ blockade in mice bearing irradiated $4T1^{shActA}$-tumors did not further improve tumor control but did improve priming with better production of IFNγ by CD8 T cells in recognition of the tumor derived epitope gp70. Surprisingly, inhibiting TGFβ or actA increased intratumoral Tregs, an effect that was reversed when both TGFβ and actA were inhibited. RT alone, as expected, increased Tregs, and the increase was markedly larger in the presence of 1D11 or actA knockdown. In marked contrast, when both TGFβ and actA were inhibited Tregs numbers decreased in irradiated tumors below baseline (Control: 11.6%; 1D11: 26.2%, shActA: 21%; 1D11+shActA: 13.6%; RT: 15.7%; RT+1D11: 27.5%; RT+shActA: 30.3%; RT+1D11+shActA: 7.9% of Tregs).

These data suggest a complex regulation of Tregs in the tumor by TGFβ and actA. Combined blockade of TGFβ and actA during RT may be required to optimize the ability of RT to induce in situ tumor vaccination and anti-tumor immune responses capable to achieve systemic control of metastatic disease (abscopal effect).

POSTER PRESENTATION – 2015 Radiation Research Society Annual Meeting.

Abstract : Radiation-induced DNA-damage response drives the secretion of activin A by tumor cells fostering immunosuppression in breast cancer.

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Activin A (actA) is a member of the transforming growth factor beta (TGFβ) superfamily. Recent evidence suggests that actA may facilitate tumorigenesis in the context of impaired growth-inhibitory response by suppressing immunity in the tumor microenvironment (Loomans et al., Cancers (Basel). 2014). Radiotherapy (RT) has the ability to convert a tumor into an in situ vaccine but its effect is suboptimal due to concomitant activation of immunosuppressive signals. Double strand DNA damage has been shown to induce actA mRNA and protein (Fordyce et al., Clin Cancer Res 2010). Therefore, we hypothesize that induction of actA by RT might be a key factor limiting activation of anti-tumor immunity.

To test this hypothesis, 4T1 mammary carcinoma cells were engineered to express a doxycycline (dox) inducible shRNA silencing inhibin A (Inhba, gene encoding for actA) ($4T1^{shInhba}$). $4T1^{shInhba}$ or its non-silencing control ($4T1^{shNS}$) were exposed to single dose (6Gy, 8Gy, 12Gy and 20Gy) RT to determine Inhba expression by qPCR as well as secretion of actA by ELISA. To determine if ataxia telangiectasia mutated (ATM) pathway in the DNA damage response (DDR) control the expression of actA, derivatives with selective inducible knockdown of ATM were also generated ($4T1^{shATM}$). $4T1^{shInhba}$, $4T1^{shATM}$ and $4T1^{shNS}$ were injected s.c. in syngeneic BALB/c mice on day 0. Knockdown of ATM and Inhba genes was induced by dox at day 8. Tumors were irradiated in 6 Gy fractions on days 13, 14, 15, 16 and 17. Mice were euthanized at day 22 for analysis and at day 28 evaluation of immune cells infiltration into the tumor.

RT upregulated actA expression and secretion by 4T1 cells. Secreted actA promoted CD4 T cells conversion into regulatory T (Tregs) cells. Knockdown of ATM abolished both Inhba expression and actA secretion by tumor cells after RT in vitro. This resulted in a decrease of immunosuppression by reducing Tregs recruitment into the tumors and increasing radiosensitivity in both $4T1^{shInhba}$ and $4T1^{shATM}$ tumor-bearing animals compared to control.

These data suggest that DDR drives actA secretion, which will attenuate the responsiveness of the immune system after RT exposure. Inhibition of actA during RT may promote self-immunization and achieve systemic control of metastatic disease.

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Journal for ImmunoTherapy of Cancer

POSTER PRESENTATION Open Access

TGFβ and activin A control regulatory T cells in irradiated tumors

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RT significantly increased actA secretion by 4T1 cells (pActA gene expression was upregulated in irradiated 4T1-tumors but was significantly higher in mice treated with 1D11 and RT+1D11. Neither in vivo 1D11 nor *ActA* gene knockdown by themselves affected tumor growth. However, each intervention significantly improved tumor control achieved by RT. TGFβ blockade in mice bearing irradiated $4T1^{shActA}$-tumors did not further improve tumor control but did significantly increase IFNγ production by CD8+ T cells in response to a tumor-specific antigen. Surprisingly, inhibiting TGFβ or actA increased intratumoral Tregs. The increase in Tregs induced by RT was markedly larger in the presence of 1D11 or actA knockdown. In marked contrast, when both TGFβ and actA were inhibited Tregs numbers significantly decreased below baseline in irradiated tumors (Control: 11.6%; 1D11: 26.2%, shActA: 21%; 1D11+shActA: 13.6%; RT: 15.7%; RT+1D11: 27.5%; RT+shActA: 30.3%; RT+1D11+shActA: 7.9% of Tregs).

These data suggest a complex regulation of Tregs in the tumor by TGFβ and actA. Combined blockade of TGFβ and actA during RT may be required to optimize activation of anti-tumor T cells induced by RT.

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